

EXHIBIT K

**UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY**

**IN RE VALSARTAN, LOSARTAN,
AND IRBESARTAN PRODUCTS
LIABILITY LITIGATION**

THIS DOCUMENT RELATES TO ALL CASES

MDL NO. 2875

Civil NO. 19-02875 (RBK/JS)

**RULE 26 EXPERT REPORT OF
DIPAK PANIGRAHY, MD**

Date: July 6, 2021



Dipak Panigrahy, M.D.

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QUALIFICATIONS

As an academic scientist, I am committed to research efforts that are focused on preventing and finding a cure for cancer. I am motivated and dedicated to discovering innovative approaches to answering important biological questions that impact carcinogenesis in humans. My scientific expertise has also extended to providing consulting services to cancer researchers in academia and industry.

I was accepted into medical school while still attending high school, graduated from the combined BA/MD program at Boston University Medical School and earned my M.D. degree in 1994. During my time in this medical program, I attended surgery with Dr. Roger Jenkins, who performed the first liver transplant in New England. From 1994 to 1996, I trained in a surgical residency at the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (UMDNJ-RWJ). Throughout my medical and post-doctoral training, I was mentored by Dr. Judah Folkman, who pioneered the field of angiogenesis, which is the growth of new blood vessels. Tumor growth is dependent on this important process of new blood vessel growth in the body¹. During my 15+ years in the Folkman laboratory, I assisted in establishing advanced angiogenesis and cancer animal models, which today serve as the foundation for studying mechanisms of cancer causation and pathogenesis in the cancer associated angiogenesis field world-wide. Abnormal blood vessel growth, either excessive or insufficient, plays an important role in many deadly and debilitating conditions, including cancer, age-related blindness, skin diseases, diabetic ulcers, cardiovascular disease, and stroke. By leading most of the *in vivo* tumor studies in the Folkman laboratory, I acquired a uniquely broad proficiency in synthesizing and using many animal tumor models to study processes affecting the initiation and promotion of cancer, including inflammation, angiogenesis, oxidative stress, immunosuppression, and apoptosis

(cell death). I also developed a comprehensive expertise in testing anti-cancer drugs and critically evaluating mechanisms of cancer causation^{2,3}. My first publication in the Folkman laboratory demonstrating a critical role of tumor angiogenesis as a critical driver of cancer was featured on the cover of the high impact journal, *The Journal of Clinical Investigation*, in 2002².

Over the past two decades, I have developed an expertise in tumor dormancy and the mechanisms of tumor dormancy escape. Tumor dormancy is defined as a period of time in which cancer cells do not grow, remain “quiet”, or where there is a balance between proliferation and death due to immunological clearance or lack of angiogenesis. My laboratory and colleagues demonstrated that the endothelium and certain stromal cells secrete signaling lipids called epoxyeicosatrienoic acids (EETs) that potently stimulate angiogenesis, multi-organ metastasis, and tumor dormancy escape (Panigrahy *et al. The Journal of Clinical Investigation* 2012⁴, voted by Faculty of 1000 as one of the top 2% of publications in biology and medicine). Based on this discovery, as the principal investigator, I was awarded a substantial grant funded by the National Institutes of Health (NIH) and the National Cancer Institute (NCI), to study EETs in cancer and metastasis. Furthermore, our laboratory and colleagues discovered that EETs play a critical role in stimulating organ and tissue regeneration (Panigrahy *et al., Proceedings of the National Academy of Science*, 2013)⁵. As part of the NCI’s Provocative Questions project, I, as the principal investigator, was awarded another multi-million-dollar grant funded by the NIH and NCI to study endogenous anti-inflammatory lipid autacoids and their role in inflammation resolution in cancer.

My laboratory has won over 50 awards for our studies on the key characteristics of carcinogens (e.g., inflammation, oxidative stress, immunosuppression, apoptosis, and angiogenesis) in cancer. In 2015, I was awarded the American Society of Investigative Pathology (ASIP) Cotran Early Investigator Award and a Young Investigator Award at the 14th International

Conference on Bioactive Lipids in Cancer, Inflammation, and Related Diseases (Budapest, Hungary). Since 2015, I have held a Visiting Professorship at Khon Kaen University in Thailand. My laboratory focuses on targeting inflammation in cancer via lipids that are ideally suited for translation into the clinic for the treatment of cancer patients. I have chaired over ten symposiums and given over 70 invited lectures at various local/national/international meetings over the past decade. I continue to play an active role in bringing together the fields of bioactive lipids and cancer biology through the organization of meetings and editorial work. I was the top candidate in an international search for a research faculty member in the Center for Vascular Biology Research at the Beth Israel Deaconess Medical Center and Harvard Medical School, where I am currently an Assistant Professor of Pathology.

Since 2018, my laboratory has published seven original research papers in various high-impact journals, which is a testament to my laboratory's world-leading expertise in helping pioneer the field of inflammation resolution in cancer. My laboratory's scientific discoveries include demonstrating that endogenous anti-inflammatory and pro-resolution lipids, such as resolvins, and dual COX-2/sEH inhibitors, stimulate the resolution of inflammation in cancer:

- I. **Sulciner *et al.* 2018. Resolvins suppress tumor growth and enhance cancer therapy** *Journal of Experimental Medicine*. **215:115-140⁶**. Featured with related Insight commentary in *Journal of Experimental Medicine*: Resolving the dark side of therapy-driven cancer cell death. Voted by Faculty of 1000 as one of the top 2% of publications in biology and medicine. Featured in Science, EurekAlert, Genetic Engineering & Biotechnology News, ecancernews, Medical News, MedIndia, Newswise, ALN Magazine, Stat News, Health Medicine Network, Science Newslane, Medical News Today, El Economista, BioCentury, MedPage Today, and Boston Globe.

- II. **Gartung *et al.* 2019. Suppression of chemotherapy-induced cytokine/lipid mediator surge and ovarian cancer by a dual COX-2/sEH inhibitor. *Proceedings of the National Academy of Sciences*. 116:1698-1703⁷.** Featured in Medical Xpress, EurekAlert, Entomology & Nematology UC News, Bug Squad Blog, and The California Aggie.
- III. **Gilligan *et al.* 2019. Aspirin-triggered pro-resolving mediators stimulate resolution in cancer. *Proceedings of the National Academy of Sciences*. 116; 6292-6297⁸.** Featured in *Harvard Medical School News & Research*: Mitchell J, A daily dose.
- IV. **Panigrahy *et al.* 2019. Pre-operative stimulation of resolution and inflammation blockade eradicates micrometastases. *The Journal of Clinical Investigation*. 17:130; 2974-2979⁹.** Featured with Editor's pick and related commentary in *The Journal of Clinical Investigation*: Dampening the fire to prevent surgery- and chemotherapy-induced metastasis, and *Research Watch* in *Cancer Discovery*.
- V. **Chang *et al.* 2019. Chemotherapy-generated cell debris stimulates colon carcinoma tumor growth via osteopontin. *The FASEB Journal* 33(1):114-125¹⁰**
- VI. **Fishbein *et al.* 2020. Resolution of eicosanoid/cytokine storm prevents carcinogen and inflammation-initiated hepatocellular cancer progression. *Proceedings of the National Academy of Sciences*. 1;117(35):21576-21587¹¹.** *Fishbein et al.* showed for the first time that simultaneously blocking two lipid pathways prevents cancer growth in mice caused by the carcinogen aflatoxin B₁. Over 4 billion people world-wide are at risk for cancer because of this carcinogen in their food.
- VII. **Deng *et al.* 2021. Resolution of debris-stimulated metastatic hepato-pancreatic cancer via combined soluble epoxide hydrolase and EP4 inhibition. *Proceedings of the National Academy of Sciences (in press)*.**

My recent commentary on COVID-19 is highly relevant to inflammation and cancer: Panigrahy *et al.* 2020. Inflammation resolution: a dual-pronged approach to averting cytokine storms in COVID-19¹² was the most read publication of *Cancer & Metastasis Reviews* (Springer Nature publishing group) in 2020 and has over 18,000 downloads to date. Based on my expertise, my colleagues and I have published several high impact review articles focused on the role of chemical carcinogens in causing cancer including Fishbein *et al.* 2021. Carcinogenesis: Failure of resolution of inflammation? *Pharmacology & Therapeutics*. 218:107670¹³; Panigrahy *et al.* 2021. Resolution of inflammation: an organizing principle in biology and medicine. *Pharmacology & Therapeutics*. April 26:107879¹⁴; and Sulciner *et al.* 2018. Targeting lipid mediators in cancer biology. *Cancer & Metastasis Reviews* 37 (2-3):557-572¹⁵. My laboratory collaborates with a number of pharmaceutical companies to advance anti-inflammatory drugs into clinical development to treat various inflammatory cancer types including pancreatic, colorectal, liver, lung, and ovarian cancer. We study the mechanisms of action of inflammation-modulating drugs, such as PPAR antagonists and resolvins, in experimental cancer models, and develop methods to translate these molecules to benefit cancer patients. Thus, I have helped pioneer a new field of research of the role of inflammation resolution in cancer.

My hourly rate for work on this litigation is \$500/hour. I have testified twice at deposition hearings including in the case of In Re Actos Products Liability Litigation (United States District Court for the Western District of Louisiana) and once at the trial of Decou and Iorio v. Takeda Pharmaceuticals America, Inc. in 2015 (District Court in Las Vegas, Nevada).

PURPOSE

I was retained by the Plaintiffs' Counsel to review the available scientific evidence regarding N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), and form

opinions on whether the NDMA and NDEA in valsartan-containing drugs could cause cancer or increase the risk of cancer in humans, and what the biologically plausible mechanisms of action may be. I was also asked to provide an opinion as to the types of cancer, if any, that can be caused by NDMA and NDEA found in contaminated valsartan drugs, and the range of latency periods for any such cancer associated with the NDMA and/or NDEA found in contaminated valsartan.

In forming opinions on these issues, I relied on my background, education, training, laboratory experience, and over three decades of researching cancer biology, carcinogenesis, the tumor microenvironment, and the role of chemicals in causing cancer and their carcinogenic mechanisms. I also reviewed materials, including published literature, clinical study reports and internal company documents. A list of materials considered in forming my opinions is included at the end of this report. I have formed the following opinions, all of which are stated to a reasonable degree of medical and scientific certainty:

SUMMARY OF EXPERT OPINIONS

- I. NDMA and NDEA are potent human carcinogens.
- II. There are biologically plausible mechanisms to support carcinogenesis from exposure to NDMA and/or NDEA.
- III. NDMA and NDEA are genotoxic and function as tumor initiators. NDMA and NDEA can cause irreversible changes to DNA that lead to cancer and increase cancer risk.
- IV. NDMA and NDEA also function as tumor promoters. NDMA and NDEA stimulate tumor progression via direct action on tumor cells (e.g., cell autonomous processes) and the tumor microenvironment.

- V. NDMA and NDEA cause human cancers including: colorectal/intestinal, esophageal/pharyngeal, gastric, kidney, liver, lung, pancreatic, prostate, bladder and blood (e.g., lymphoma, leukemia and multiple myeloma).
- VI. Human cumulative exposure to greater than 96 nanograms per day of NDMA or greater than 26.5 nanograms per day of NDEA increases one's risk of developing cancer.
- VII. The latency period between NDMA or NDEA exposure and cancer induction including tumor dormancy escape can range from 6 months to 10+ years or more.
- VIII. Recent exposure to potent carcinogens, like NDMA and NDEA, is more likely to be a significant factor in cancer causation than historical exposures.
- IX. Based on valsartan dosing, the levels of NDMA and NDEA reported in contaminated valsartan, and the time frame over which the contamination occurred, it is medically and scientifically plausible that a number of users of contaminated valsartan have already or will develop clinical cancer.
- X. The most plausible mechanisms by which NDMA and NDEA cause cancer in humans include 9 of 10 key characteristics of carcinogens: (1) metabolic activation, (2) genotoxicity, (3) alteration of DNA repair, (4) induce epigenetic alterations (5) oxidative stress, (6) chronic inflammation, (7) immunosuppression, (8) cell immortalization, and (9) alteration of cell proliferation, cell death, and angiogenesis (the growth of new blood vessels).
- XI. Exposure to NDMA and/or NDEA at the levels present in the contaminated valsartan can initiate and critically promote cancer growth.
- XII. Continued exposure to NDMA and/or NDEA can cause an existing cancer to grow, metastasize and otherwise interfere with cancer therapy.

XIII. Because of the multiple toxicities and potential tumor-promoting activity associated with standard cancer therapies (e.g., surgery, chemotherapy, and radiation), people who develop and are treated for NDMA- and/or NDEA-induced cancer remain at increased risk for tumor recurrence and developing secondary cancers and will require life-long monitoring.

VALSARTAN-RECALL

Valsartan is a prescription medication used by millions of Americans to treat hypertension (high blood pressure) and congestive heart failure, as well as to prevent heart attacks and strokes. Valsartan works by inhibiting angiotensin II, a biological mediator that causes contraction of blood vessel walls resulting in hypertension¹⁶. Generic drugs such as valsartan are sold when the brand-name version of the drug comes off patent, and these generic equivalents are designed and required to be of equal quality and equal safety. Valsartan is manufactured in four tablet dosages: 40 mg, 80 mg, 160 mg and 320 mg¹⁷. The recommended dose for adults is initially 80 mg or 160 mg daily with a targeted maximum dose of 160 mg twice daily. Heart failure patients are usually started on two 40 mg tablets daily. Heart attack patients often take half of one 40 mg tablet twice daily before increasing the dose.

On July 5, 2018, the European Medicines Agency (EMA) reviewed medicines containing valsartan following detection of an impurity, N-nitrosodimethylamine (NDMA), a probable human carcinogen, in medicines from Zhejiang Huahai Pharmaceutical Co Ltd, Linhai, China. Immediately following the EMA's review, 22 European countries recalled approximately 2,300 batches of valsartan products, while Hong Kong recalled 5 drug products from 2 companies and Canada recalled drug products from 5 companies. The Drug Regulatory Authority of Pakistan on July 12, 2018, recalled valsartan-containing drugs from 9 manufacturers becoming the first

developing country to announce the recall separately as a precautionary measure to protect patient health.

On July 13, 2018, the FDA announced a voluntary recall of several lots of valsartan-containing generic medications due to contamination with NDMA. On July 27, 2018, the FDA issued a press release explaining the reason for its concern regarding the presence of NDMA found in valsartan-containing drugs. The statement provided: the Environmental Protection Agency classified NDMA as a probable human carcinogen “based on the induction of tumors at multiple sites in different mammal species exposed to NDMA by various routes.” In September 2018, the FDA announced that its latest testing of recalled products showed an additional impurity, N-nitrosodiethylamine (NDEA), which is another potent cancer-causing chemical. On September 13, 2018, the EMA also reported that the NDEA impurity had been identified in valsartan. To date, 624 lots of angiotensin II receptor blockers containing valsartan or in combination with other active drug substances have been recalled in the United States as a result of containing unacceptably high levels of nitrosamines. Please see <https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan>.

The FDA conducted an investigation into the presence of NDMA and NDEA in valsartan-containing products which revealed NDMA contamination levels as high as 20.19 micrograms (20,190 nanograms) per tablet and NDEA contamination levels as high as 1.31 micrograms (1,310 nanograms) per tablet¹⁸. See <https://www.fda.gov/drugs/drug-safety-and-availability/laboratory-analysis-valsartan-products>

Following the recall by the defendants, the NDMA and NDEA testing demonstrated even higher levels of contamination. For example, Zhejiang Huahai Pharmaceutical (ZHP) batches of

valsartan have been recorded to contain 188.1 ppm of NDMA (See ZHP 118/SOLCO00028261) which equates to 60,192 nanograms in a 320 mg tablet. The NDEA recorded in a Torrent batch reached 16.93 ppm (See LP1337 – TORRENT-MDL2875-00135398) which equates to 5,417 nanograms in a 320 mg valsartan tablet. There were various levels of contamination reported across the different manufacturers. Torrent reported testing levels of NDMA in approximate range of 0.37 to 125.15 ppm. (TORRENT-MDL2875-00366172), and NDEA in the range from 0.23 to 16.93 ppm (TORRENT-MDL2875-00135398). Mylan reported NDMA in the approximate range from 0.01 to 0.09 ppm, and NDEA ranging from 0.1-1.57ppm. (MYLAN-MDL2875-00895544). ZHP reported NDMA in the approximate range of 3.4 to 188.1 ppm for one process and ranging from ND to 73.9 for another process (ZHP00079920-9940). ZHP reported levels of NDEA ranging approximately from 0.03 to 42.14 ppm for one process, and from 0 to 4.23 ppm for another process (PRINSTON0075857-858). Hetero reported NDMA levels ranging from approximately 0.82 to 2.69 ppm. (HETERO_USA000025250-251). Auro-MDL-2875-0104586). Teva reported levels of NDMA in the approximate range of 0.02 to 31.3 ppm. (TEVA-MDL2875-00069442; TEVA-MDL-00693423), and NDEA levels ranging from 0.02-0.50 ppm. (TEVA-MDL2875-00048605). Aurobindo reported NDMA approximately ranging from 0.106 to 0.129 ppm, and NDEA ranging from 0.028 to 1.508 ppm. (Auro-MDL-2875-0093561).

The FDA recognized the danger of valsartan tablets containing NDMA and/or NDEA and set strict daily acceptable intake limits for NDMA (0.3 ppm) and NDEA (0.083 ppm) in valsartan. Based on the maximum valsartan dose of 320 mg, this equates to 96 nanograms per day of NDMA and 26.5 nanograms per day of NDEA. The detectable levels of NDMA and NDEA found in the contaminated valsartan tablets are much higher than these values. For example, the batch containing 188.1 ppm of NDMA is 627 times higher than the acceptable intake established by the

FDA. The batch containing 16.93 ppm of NDEA is 204 times higher than the acceptable intake established by the FDA.

The FDA derived the daily acceptable intake values for NDMA and NDEA in valsartan from compound-specific animal toxicological data. They used the TD₅₀ estimate of the lowest dose, which is 95% certain to cause no more than a 10% cancer incidence in rodents, as the point of departure for the calculation of excess cancer risk (the TD₅₀ is a toxicology term that means the median toxic dose of a substance in which toxicity occurs in 50% of a species). The TD₅₀ is a measure of carcinogenic potency that can be used to compare carcinogens. The TD₅₀ listed for NDMA is 0.096 mg/kg/day calculated as a harmonic mean from all positive studies in rats including data from the Peto et al. (1991) study¹⁹. The extrapolation to the excess risk level for cancer is performed by linear back extrapolation to the dose theoretically causing a 1:100,000 risk by dividing the TD₅₀ by 50,000 (50% or 0.5 x 100,000). For NDMA this translates into a dose of 1.92 ng/kg/day. For a person with a body weight of 50 kg, this would result in an acceptable index (AI) level of 96 ng/day (50 x 1.92 ng) and the 96 ng/day corresponds to 0.3 ppm in a 320 mg Valsartan tablet. The same methodology was followed for NDEA resulting in an acceptable intake of 26.5 ng/day. The FDA has determined that an acceptable intake is a daily exposure to compounds such as NDMA or NDEA that approximates a 1:100,000 cancer risk after 70 years exposure. *The calculated acceptable intake for NDMA is based on methods described in the ICH Guidance M7(R1) Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk* (<http://wcms-internet.fda.gov/files/drugs/published/M7-R1-AssessmentAndControlOfDNA-Reactive-Mutagenic-ImpuritiesInPharmaceuticalsToLimitPotentialCarcinogenicRisk-Guidance.pdf>)

According to Haber's rule, a fundamental principle in toxicology, concentration (C) x time (T) = constant (k); therefore, the carcinogenic effect is based on both dose and duration of exposure²⁰. This is generally accepted to be used for mutagenic carcinogens and therefore considered appropriate to apply to NDMA and NDEA. The total dose taken over time (dose x time) produces a fixed level of effect; thus, determining the risk associated with the exposure. Applying this conservative principle, the cumulative dose acceptable for a lifetime is equivalent to the daily acceptable intake limit multiplied by the duration of a lifetime (70 years is generally accepted for this), which is 25,550 days. The FDA established 96 nanogram daily acceptable intake limit of NDMA corresponds to a lifetime dose of 2,453 micrograms. The FDA established 26.5 nanogram daily acceptable intake limit of NDEA corresponds to a lifetime dose of 677 micrograms.

CARCINOGENESIS: BACKGROUND

The widely accepted meaning of the term "*chemical carcinogenesis*" that is used in the IARC monographs, is the chemical induction of cancer (or neoplasm) that is not usually observed at baseline^{13,21}. A neoplasm is defined as an abnormal growth of cells in the body, also referred to as a tumor. Carcinogenesis is a multi-step process in which normal cells are transformed into cancer cells by acquiring various properties that allow them to proliferate unabated and form tumors¹³. Chemical carcinogenesis can be divided into three stages: initiation, promotion, and progression²².

A carcinogen is an agent with the capacity to cause cancer in humans. If something causes cancer it is a carcinogen. A *carcinogen* is defined as a chemical substance, or a mixture of chemical substances, after inhalation, ingestion, dermal application or injection which induces cancer, increases its incidence, or shortens the time to tumor occurrence (e.g., latency) at any dosage level

by any route in any species as compared to control^{13,23}. Carcinogens may be natural, such as aflatoxin, which is produced by a fungus and sometimes found on stored grains; or manmade, such as asbestos or tobacco smoke. Carcinogens work by interacting with a cell's DNA and inducing genetic mutations. Many human carcinogens act via multiple mechanisms causing various biological changes in the multistage process of carcinogenesis. Mechanisms by which carcinogens induce and stimulate cancer include genotoxicity, DNA damage, inflammation, oxidative stress, immunosuppression, angiogenesis, acute or chronic injury, and subsequent regenerative proliferation via cell death^{13,24,25}.

The link between smoking, perhaps the most well-known human carcinogen, and cancer is very clear. Smoking causes at least 15 different types of cancer, including two of the most common, lung and intestinal cancer (colon/rectum), as well as mouth, pharynx (throat), nose and sinuses, larynx (voice box), esophagus (food pipe), liver, pancreas, stomach, kidney, ovary, bladder, cervix, and some types of leukemia²⁶. Smoking causes cancer via multiple mechanisms including metabolic activation, DNA adduct formation, inflammation, and mutation of critical genes²⁶. There is no safe level of smoking. Smoking releases harmful chemicals which enter the lungs and spread across the entire body²⁶. Thus, being smoke free can prevent over 15 types of cancer. Importantly, the 1964 Surgeon General's Report linking cigarette smoking and lung cancer has had an enormous positive effect on public health in the U.S. Since then, male smoking prevalence decreased from 51.1% to the current 21.6% while prevalence in females diminished from 33.3% to 16.5%²⁶. These facts clearly demonstrate the critical importance of public health stances on avoiding carcinogens such as tobacco.

More than 70 carcinogens have been classified by the International Agency for Research on Cancer (IARC) as having sufficient evidence for carcinogenicity in either laboratory animals

or humans²⁷. These include polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, volatile nitrosamines, alcohol, aromatic amines, aldehydes, and volatile hydrocarbons such as benzene¹³. Many of these carcinogens induce multiple cancer types.

Although epidemiology and studies with human tissues or cells are relevant to carcinogen exposure in humans, the mechanistic studies underlying carcinogenesis are focused in animal models for obvious ethical considerations. It would be unethical to use humans as “test subjects” to evaluate the carcinogenic activity of potential carcinogens. Importantly, laboratory animals are routinely utilized to study the mechanism of action of carcinogens and mimic cancer in humans because of their genetic, physiologic, biochemical, and metabolic similarities to humans, large sample size, reproducibility, and feasibility to generate various cancers²⁸. Recent exposure to potent carcinogens, like NDMA and NDEA, is more likely to be a significant factor in cancer causation than historical exposures of other agents²⁹. Importantly, a single dose of NDMA causes cancer in mice (32 different strains or substrains), rats (10 different strains or substrains), mastomys (praoys) natalensis, rainbow trout, and Syrian golden hamsters²⁹. Similarly, a single dose of NDEA causes cancer in mice (69 different strains or substrains), rats (10 different strains or substrains), gerbils, *Rivulus maroratus*, (fish), and Syrian golden hamsters²⁹.

The mechanisms of action of carcinogens have traditionally been classified as genotoxic and/or nongenotoxic. A genotoxic carcinogen is defined as a chemical that causes cancer by directly altering the genetic material of target cells, while nongenotoxic carcinogens are chemicals that can induce cancer by mechanisms not related to direct genetic damage. Studies now show that classification of carcinogens into genotoxic initiators and nongenotoxic promoters is an oversimplification of the complex processes involved in chemical carcinogenesis.

Cancer causation and mechanistic studies can be demonstrated in animal models after exposure to potential carcinogens. Chemicals that are carcinogenic via genotoxicity to rodents are presumed to be carcinogenic to humans unless proven otherwise²⁸. Because genotoxic carcinogens are mutagenic and may interact with DNA to produce irreversible genetic changes in target organ cells, they may exhibit no dose threshold for their carcinogenic potential³⁰⁻³². While carcinogen-induced DNA damage can cause cancer, mechanisms such as inflammation and angiogenesis which do not directly damage DNA are also critical to cancer progression^{1,11,33}.

Carcinogens have recently been described by their 10 key characteristics that encompass genotoxic and nongenotoxic activity^{24,25}. In 2019, the key characteristic profiles were determined for carcinogens based upon human and animal *in vivo* and *in vitro* studies. *In vivo* means taking place inside the body while *in vitro* means taking place in culture or a petri dish outside the body. The most prevalent key characteristic is that it "is genotoxic", followed by "alters cell proliferation, cell death, or nutrient supply" and "induces oxidative stress"³⁴. Most agents exhibited several of the 10 key characteristics, with an average of four characteristics per agent, a finding consistent with chemical-induced cancer development in humans occurring via multiple pathways³⁴.

METHODOLOGY EMPLOYED

To formulate an opinion as to whether the NDMA and/or NDEA contamination in valsartan-containing drugs can cause and increase the risk of cancer in humans, I used the same methodology I would use if conducting clinical work or research for scientific publication. This methodology is consistent with what is followed by cancer research scientists when evaluating whether a chemical agent is a human carcinogen and whether exposed individuals are at risk of developing cancer because of their exposure. I have reviewed relevant published scientific studies and medical literature, reports and documents produced in the process of litigation, and various

websites that were relevant to the refinement or extension of my professional opinions. This evaluation of whether the NDMA and NDEA in valsartan-containing drugs can cause cancer in humans required the analysis of the scientific data from animal cancer studies, animal and human tissue and cell studies investigating the mechanisms through which NDMA and NDEA cause cancer, and studies of human populations exposed to NDMA and NDEA. There are numerous studies that provide valuable information regarding NDMA and NDEA as carcinogens as both are used as a first step in experimental animal cancer studies. My opinions are not based solely on my education and expertise, but also are based upon peer-reviewed publications, studies, and scientific evidence.

Peer-reviewed publications are an important part of the scientific investigation process. In the scientific review process, a group of scientists complete a study and submit their findings in the form of an article to a scientific journal for publication. The journal's editors send the article to several other scientists who usually work in the same or similar field (e.g., the "peers" of peer review). Those reviewers provide feedback on the article and tell the editor whether or not they think the study is of high enough quality to be published and which new experiments should be performed as appropriate controls prior to publication. The authors may then revise their article and resubmit it for consideration to be approved by the peer reviewers and journal editors. Only articles that meet scientific standards (e.g., acknowledge and build upon other work in the field, rely on logical reasoning and well-designed studies, and back up claims with scientific evidence) are finally accepted for publication. Given this rigorous review process, original peer-reviewed publications of new scientific studies are given greater weight in the scientific community than non-original articles such as reviews.

There are three principal types of studies used to determine if a chemical is carcinogenic

to humans when there have not been human randomized control trials (RCTs). In blinded RCTs, both the investigator and study participants are blinded to treatment or placebo assignment so any differences in incidence of outcome can be concluded to be a consequence of the exposure itself (e.g., causative). However, there are no RCTs which measure the outcome of cancer associated with either NDMA or NDEA in humans. Because cancer causes death, it is unethical to ask for consent from a healthy human volunteer to be tested with NDMA or NDEA knowing that they are both probable human carcinogens. An important step in medical students becoming doctors is to uphold the Hippocratic Oath, which is held sacred by physicians who swear to uphold specific ethical standards and promise to abstain from doing harm to their patients. “Primum non nocere” is the Latin phrase that means “first, do no harm.” This is a commonly taught principle in medicine and healthcare. Thus, no doctor should knowingly subject a human to NDMA or NDEA in a human clinical trial. Instead, scientists must rely on animal cancer studies as well as mechanistic studies with animal and human tissue or cells, and human studies with unintentional exposures which contain NDMA (e.g., rubber dust or diet).

As such, to answer the question whether the NDMA and NDEA in valsartan-containing drugs can cause cancer in humans, I reviewed, analyzed and synthesized: 1) animal cancer studies; 2) mechanistic studies of biological activity in animal tissue and cells; 3) mechanistic studies of biological activity in human tissue and cells; and 4) in the absence of RCTs, dietary and occupational epidemiology studies with data relating to quantified exposures to NDMA and NDEA and the risk of cancer.

It is accepted practice to utilize animal studies to determine cause and effect between toxic chemicals and cancer²⁸. Qualitative extrapolation in toxicology rests upon the principal that a compound that causes an effect in one mammalian species causes it in another. There are three

essential tenets on which the toxicologic discipline rests: a) the dose makes the poison; b) a chemical agent tends to produce a specific pattern of biological activity; and c) the toxic responses in laboratory animals are useful predictors of toxic responses in humans. To determine whether a chemical can cause cancer in humans, it is critical to use scientific evidence to quantify the extent of chemical-induced tumor formation in experimental animals. When evaluating these studies, it is important to incorporate our current understanding of carcinogenesis in laboratory animals and consider: 1) the induction of tumors in multiple species (e.g., multiple tumors in site-specific manner); 2) independent studies by different investigators; 3) the occurrence of common versus uncommon neoplasia (cancer); 4) latency in tumor induction; 5) metastases (development of secondary, distant tumor sites); and 6) the presence or absence of dose-response relationships.

Long-term chemical carcinogenesis bioassays in experimental animals remain the most globally accepted method of identifying potential human carcinogens^{28,32,35-37}. A bioassay is a biochemical test that determines the activity of a chemical by measuring its effect on an organism, tissue, cell, enzyme or receptor preparation compared to a standard preparation. Importantly, exposures to chemicals are tightly controlled and monitored in animal bioassays. Accordingly, in the absence of adequate testing on humans, it is biologically plausible and prudent to regard agents for which there is sufficient evidence of carcinogenicity in experimental animals as “*presumed human carcinogens*”^{28,38}. Thus, substances that cause tumors in animals are considered as *presumed or suspected human carcinogens* unless convincing evidence to the contrary is presented²⁸.

In addition to animal studies, I reviewed studies on human tissue and cells relevant to NDMA and NDEA. In general, the strongest indicator that a particular mechanism operates in humans is data from exposed humans or from human tissue and cells in combination with animal cancers studied. The objective identification of mechanistic data for consideration of biologic

plausibility in the context of epidemiology, animal bioassay, or other types of evidence (e.g., studies in model organisms or *in vitro* assays) is important when classifying agents with regard to carcinogenic hazard.

Epidemiological studies are designed to generate statistical data in support of the scientific effort to determine what, if anything, that data might reveal about a possible causative relationship or association in humans. I reviewed the available human epidemiology studies that quantified the exposure to NDMA or NDEA which included contaminated valsartan related studies, dietary studies, and occupational studies. There are no RCTs to review as it is not ethically permissible to conduct human clinical trials with carcinogens such as NDMA and NDEA.

The American Cancer Society does not determine if something causes cancer. Instead, it and other governing bodies (e.g., FDA) rely heavily on the determination of other respected agencies, such as the International Agency for Research on Cancer (IARC), United States National Toxicology Program (US NTP), and the Environmental Protection Agency (EPA). When conducting my investigation, I followed the basic steps employed in the peer-review scientific process used by scientists and IARC/NTP/EPA which included:

1. Identification of the scientific agencies that evaluate the carcinogenicity of various agents to humans such as IARC, NTP, and EPA, and conducted a search for their scientific review and evaluation of NDMA and NDEA.
2. Identification of the relevant scientific publications through initial comprehensive searches of literature contained in authoritative biomedical databases (e.g., PubMed). These literature searches were designed to find relevant studies to determine whether NDMA and/or NDEA causes cancer in humans, causes cancer in experimental systems, and/or exhibits key characteristics of established human carcinogens (in humans or in

experimental systems). Searches were designed to identify relevant studies regardless of whether they supported or did not support a causal relationship between NDMA and/or NDEA and cancer.

3. Screening the retrieved literature for inclusion based on title and abstract review. Exclusion of the studies occurred if they were not related to NDMA and NDEA (or a metabolite of the agent), or if they reported no original data (e.g., review articles).
4. Evaluation of the quality of the included studies was based on considerations such as design, methodology, the experimental conditions under which NDMA or NDEA was tested including route of administration and exposure, species, strain, sex, age, etc., the dose, duration and frequency of treatment with NDMA or NDEA, how tumor response was measured, the experimental animal species and strains evaluated, numbers of animals per group, whether animals were allocated randomly to groups, whether there was sufficient mechanism of action analysis, whether the data was reported and analyzed adequately, the reproducibility of the study, the spectrum of neoplastic response (from benign neoplasia to multiple malignant tumors), tumor sites evaluated, and dose-response.

Importantly, I was able to identify over 120 peer-reviewed publications by over 100 different laboratories worldwide over the past 60 years which clearly demonstrate that NDMA and NDEA cause cancer in a wide variety of animal models in over 10 tumor-types. This is critical confirmation of the overwhelming scientific evidence that NDMA and NDEA are human carcinogens which cause cancer via multiple biologically plausible mechanisms.

I also performed an analysis using Bradford Hill (1965)³⁹ criteria of causation when assessing the causal relationship between NDMA and NDEA and human cancer, and weighing the

evidence. These criteria are the strength of association, consistency across populations, specificity, temporality, dose-response (biologic gradient), plausibility, coherence, experiment, and analogy. I found these factors to be instructive when evaluating the available evidence and formulating my opinion as to whether NDMA and NDEA cause human cancer. Furthermore, my opinions provided herein, all expressed with a reasonable degree of scientific and medical certainty, are based upon the weight of the evidence and my experience as a MD cancer research scientist for 25 years.

**ANIMAL STUDIES: HUMAN RELEVANCE OF CHEMICALS THAT CAUSE
CANCER IN ANIMALS**

The use of laboratory animals to identify carcinogenic potential of chemicals, mixtures, and other agents has a modern history of greater than 60 years from which copious scientific and public health information can be derived²⁸. Experimental evidence indicates that there are more physiologic, biochemical, and metabolic similarities between laboratory animals and humans than there are differences²⁸. These similarities increase the probability that results obtained in a laboratory setting would yield similar results for humans²⁸. Clearly, the accumulated experience in the field of carcinogenesis supports this concept²⁸.

Past approaches evaluating human relevance have focused on whether the animal response is generalizable. This involved observing tumors in multiple species, in multiple independent studies, or under different experimental conditions. It is important to elucidate the mechanism(s) involved in the induction of tumors in experimental animals, followed by an investigation of similarities and differences between experimental animals and humans to allow an informed determination about whether the mechanism(s) operating in experimental animals are also likely to be operative in exposed human populations³⁷. Thus, experimental animal studies can adequately demonstrate *causality*, while their evaluation must address the question of *human relevance*³⁷.

Experiments using animal model surrogates for humans are the most reliable means for determining potential carcinogenic hazards to people exposed to chemical carcinogens, and long-term experimental animal studies closely mimic overall human biology⁴⁰. *Animal data on the carcinogenicity of a variety of chemicals have preceded as well as predicted later epidemiological observations in humans.* In almost all cases in which human carcinogens have been tested in animals, there is at least one or more common target sites for carcinogenesis⁴¹. Cellular and molecular changes leading to cancer at these sites are expected to be similar to cellular and molecular changes seen in human cancers²⁸. ***Mechanistically, it matters less where the eventual cancer target site may be; the important observation is whether a chemical does or does not cause cancer in experimental animals***²⁸. As stated in Tomatis et al.⁴², “*There is an obvious logic in assuming that if exposure to an agent causes cancer, avoiding exposure to it prevents cancer. All chemicals known to induce cancer in humans that have been studied under adequate experimental conditions, also cause cancer in laboratory animals. Thus, reducing exposure to chemicals known or suspected to cause cancer in humans or animals will reduce chemically-induced cancer in humans.*”⁴³”

Because similar pathway of mechanisms of cancer causation of NDMA are found in animals and humans, the experimental animal studies on NDMA are valid and establish NDMA as a human carcinogen. Because a positive association has been found in experimental animals between the capability of agents to form O6-methylguanine in DNA of target tissues and their carcinogenic potency in those tissues⁴⁴, NDMA is a human carcinogen.

If cancer risks were to be assessed only from epidemiological evidence of affected individuals, then the implementation of public health decisions to reduce or eliminate exposures could require up to 30 years or more of prior exposure to provide sufficient numbers of cancer

deaths to ascertain human cancer causation. This practice would have grave human consequences. Dismissing animal carcinogenicity findings would lead to human cancer cases as the only means of demonstrating carcinogenicity of cancer-causing chemicals. This is unacceptable public health policy³⁶. ***Thus, chemicals for which there is “sufficient evidence” of carcinogenicity in test animals should be regarded for practical purposes as if they were carcinogenic to humans⁴⁰.***

Rodent models remain the most commonly used animal model for studying human disease because of their relatively low cost and maintenance requirements, rapid reproduction rates, and availability of research tools such as murine antibodies⁴⁵. Chemical-induced cancer models have provided a tremendous amount of valuable information on the underlying mechanisms of clinical disease. There are considerable molecular and cellular similarities in carcinogenic processes among mammals, including rodents and humans^{35,46,47}. Carcinogenesis is dissected into constituent steps, thereby exposing sites for intervention¹³. For those chemicals identified as carcinogenic to humans, experiments in animals have shown remarkable target organ concordance, meaning the same cancer types that chemicals cause in animals could occur in humans^{35,41,48}. For many exposures causally related to human cancer, there is a target organ in common between humans and at least one animal species, despite many inherent physiological differences⁴⁸. The experimental approach for the identification of carcinogens has an irreplaceable role in preventing the dispersal of new hazards into our environment⁴¹.

Although rodent models have been invaluable in the screening of human carcinogens and furthering the mechanistic understanding of cancer, there are circumstances in which choosing a large animal species in place of a rodent model for chemical-induced cancer is scientifically justifiable. As investigators translate the wealth of basic science information developed from rodent models, large animal models provide a number of translational advantages^{49,50}. Large

animal models more closely approximate the clinical and pathologic features of human disease. The dog, swine, and monkey have been used extensively because their anatomy and physiology are considered to be highly similar to that of human beings. Large animals have many fundamental anatomic, physiological, genomic, proteomic, immunologic, and nutritional similarities to human beings. Swine are considered to be one of the main animal species used in translational research, surgical models, and procedural training and are increasingly being used as an alternative to the dog or monkey as the choice of nonrodent species in preclinical toxicologic testing of pharmaceuticals⁵¹. There are unique advantages to the use of swine in this setting given that they share similar anatomic and physiologic characteristics involving the cardiovascular, urinary, integumentary, and digestive systems to humans⁵¹.

Animal models allow for the identification of potential public hazards²⁸. The use of rodent models to evaluate the carcinogenic potential of chemicals and other agents has a long and eventful history. Until a better, more rapid, less expensive, and more accurate and predictable assay to evaluate carcinogenesis is found, ***long-term chemical carcinogenesis bioassays*** remain the best and most globally accepted means for identifying potential human carcinogens^{28,32,36}. Nearly one-third of confirmed carcinogens in humans were identified first in experiments using laboratory animals^{42,52,53} and all chemicals known to induce cancer in humans that have been studied under adequate experimental protocols also cause cancer in laboratory animals^{42,48}, demonstrating that ***chemicals shown to unequivocally induce cancer in laboratory animals, especially in multiple species, must be considered capable of causing cancer in humans***³⁵.

N-NITROSODIMETHYLAMINE (NDMA)

Scientific Agencies That Evaluate Carcinogens

National and international health agencies provide a scientific basis for governmental and private efforts to prevent cancer by reducing exposure to known and suspected human carcinogens. These highly respected authoritative agencies have published criteria in scientific journals that are used for evaluating potential carcinogens including the World Health Organization-affiliated International Agency for Research on Cancer (*IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*), the *Report on Carcinogens* published by the United States National Toxicology Program (NTP), health risk assessments developed by the United States Environmental Protection Agency (EPA), and the list of chemicals known to cause cancer maintained by the California Environmental Protection Agency³⁷. Each program considers all relevant studies in an evaluation of carcinogenicity, including cancer studies in humans, cancer studies in experimental animals, and mechanistic data³⁷.

The identification of a potential carcinogen requires rigorous scientific evaluation involving long-term bioassays in experimental animals, human epidemiologic studies, and the careful collection of other data relevant to cancer causation mechanisms³⁸. These experts (e.g., IARC and NTP) review the available experimental scientific evidence to determine the cancer-causing potential of different substances. Under this paradigm, which is consistent with a multifactorial view of carcinogenesis, it is possible to identify carcinogens through mechanistic understanding alone, without waiting for epidemiological studies³⁷. The IARC, NTP, and other respected scientific organizations agree unanimously: *in the absence of human data, animal studies are the most definitive for assessing human cancer risks*. Dismissing animal carcinogenicity findings would lead to human cancer cases as the only means of demonstrating

carcinogenicity of environmental agents, which is an unacceptable public health policy²⁸. Animal data should not be ignored, and precautions should be taken to lessen human exposures. Thus, the following organizations determined many years ago, in the absence of NDMA epidemiology studies, that NDMA and NDEA should be regarded as human carcinogens.

**1. World Health Organization (WHO)/ International Agency for Research
on Cancer (IARC)**

The IARC is a research organization that evaluates the evidence on the causes of cancer but does not make health recommendations. Health and regulatory agencies include IARC evaluations in their consideration of actions to prevent exposure to potential carcinogens. The cancer hazard classification methodology, outlined in the IARC Preamble, is based on the systematic assembly, review and integration of evidence of cancer in humans, cancer in experimental animals and cancer mechanisms. Carcinogen identification involves the joint consideration of human epidemiologic studies, cancer bioassays, and mechanistic and other relevant data to determine whether an agent can increase the risk of cancer in humans⁵⁴.

The IARC Monographs are a series of scientific reviews that identify environmental factors that can increase the risk of cancer in humans. To date, more than 1000 agents have been classified. IARC evaluations are used worldwide by national and international health agencies to support a wide range of subsequent activities ranging from research, to risk assessment, to preventative actions. The IARC Monographs Programme identifies the causes of human cancer⁵⁵. The IARC assembles expert international, interdisciplinary Working Groups, free of conflicts of interest, to evaluate agents that have evidence of human exposure and are suspected to be carcinogens. The IARC Monographs are unique in that the critical reviews and evaluations are developed by the experts who performed the original research⁵⁴. Working Group Members are selected on the basis

of knowledge and experience, and to avoid real or apparent conflicts of interests⁵⁴. In general, for cancer in humans, cancer in experimental animals, and mechanistic evidence, only studies that have been published or accepted for publication in the openly available scientific literature are reviewed. The reliance on published and publicly available studies promotes transparency and protects against citation of premature information on scientific studies.

Although the IARC Monographs have emphasized hazard identification, important evaluations may also involve dose–response assessment. A subsequent publication may also be prepared by a separate Working Group with expertise in quantitative dose–response assessment. In many cases, the same epidemiological and experimental studies used to identify a cancer hazard can also be used to estimate a dose–response relationship. A Monograph may estimate dose–response relationships within the range of available epidemiological data, or it may compare the dose–response information from experimental and epidemiological studies.

When assessing mechanistic information, the *IARC Monograph* working groups answer the following questions; (1) is the mechanistic evidence that a chemical causes cancer weak, moderate, or strong?, and (2) is the mechanism likely to be operative in humans?³⁷ **Strong mechanistic data can play a pivotal role in the overall classification of an agent as a carcinogen when there are limited human cancer studies and they are not possible to obtain because it would be unethical to perform a study in humans (e.g., NDMA or NDEA).** The long-term objective is to critically review and evaluate published scientific evidence for all carcinogenic hazards to which humans are exposed³⁷.

Experts in mechanisms of carcinogenesis evaluate the strength of the mechanistic data and determine whether the mechanisms that cause cancer in experimental animals can also operate in humans. Through the combination of human, animal, and mechanistic evidence, a determination

is made that a particular agent is either: Carcinogenic to humans (Group 1); Probably carcinogenic to humans (Group 2A); Possibly carcinogenic to humans (Group 2B); Not classifiable as to its carcinogenicity to humans (Group 3); or probably not carcinogenic to humans (Group 4).

Importantly, NDMA was classified as a “**probable carcinogen**” by the IARC without consideration of any human epidemiology studies signifying the compelling experimental animal data as well as human tissue and cell data with NDMA. In 1978, IARC concluded that “**there is sufficient evidence of a carcinogenic effect of *N*-nitrosodimethylamine in many experimental animal species. Similarities in its metabolism by human and rodent tissues have been demonstrated. Although no epidemiological data were available (and efforts should be directed toward this end), *NDMA* should be regarded for practical purposes as if it were carcinogenic to humans.**” (IARC Evaluation 1978). The IARC considered that previously, the most exposed workers to NDMA were in the rocket fuel industry and the pesticide industry.

The IARC reached this conclusion because NDMA caused cancer in numerous species of experimental animals, at several different tissue sites, and by several different routes of exposure (e.g., oral ingestion, inhalation, intra-tracheal, subcutaneous, intramuscular and intraperitoneal). Tumors were observed in all species tested, including mice, rats, hamsters, guinea pigs, multimammate mice (genus *Mastomys*), rabbits, frogs, newts, and various fish. NDMA is carcinogenic following prenatal exposure, even when administered as only a single-dose.

2. The United States National Toxicology Program (NTP)

The NTP published the *Report on Carcinogens*, which identifies and discusses substances that can pose a carcinogenic hazard to human health and to which a significant number of persons residing in the United States are exposed³⁸. The NTP *Report on Carcinogens* lists agents “known

to be a human carcinogen” or “reasonably anticipated to be a human carcinogen.” NDMA is identified by the NTP as “**reasonably anticipated to be a human carcinogen** based on sufficient evidence of carcinogenicity from studies in experimental animals”³⁸.

3. United States Environmental Protection Agency (EPA)

The EPA assesses the health hazards of chemical contaminants present in the environment³⁸. Chemical assessments are developed through a toxicological review of the pertinent scientific literature written by U.S. EPA scientists or contractors, internal and external peer reviews, and an internal consensus review (U.S. EPA 2004). The EPA classifies NDMA as a “**B2 (probable human) carcinogen**”, based on the induction of tumors in both rodents and non-rodent mammals exposed to NDMA by various routes of administration. According to the EPA, in animal studies of various species including rats and mice, exposure to NDMA causes tumors primarily of the liver, respiratory tract, kidney and blood vessels. In fact, many federal regulations list NDMA as a “**priority toxic pollutant**”. The EPA set the permissible level of NDMA in drinking water at only 7 nanograms (seven billionths of a gram) for every liter of water.

The following excerpt details the EPA’s guidelines for carcinogen risk assessment: “*Agents that are positive in long-term animal experiments and also show evidence of promoting or carcinogenic activity in specialized tests should be considered as complete carcinogens unless there is evidence to the contrary.* (Guidelines for Carcinogen Risk Assessment: Risk Assessment Forum, U.S. Environmental Protection Agency, Washington D.C., September 1986)

4. The US Department of Health and Human Services (DHHS)

The DHHS concluded that NDMA “***is reasonably anticipated to be a human carcinogen***” after identification of tumors in the livers, respiratory tracts, kidneys, and blood vessels of

experimental animals exposed to NDMA (DHHS 2011).

5. Environment Canada and Health Canada

NDMA is considered “*highly likely to be carcinogenic to humans*” as determined by Environment Canada and Health Canada (2001). The mechanism by which NDMA causes cancer is well understood to involve biotransformation by liver microsomal enzymes, generating the methyldiazonium ion.

6. Commission de la santé et de la sécurité du travail (CSST)

NDMA is considered “*suspected carcinogenic in humans*” as determined by CSST: Commission de la santé et de la sécurité du travail (Québec’s workman compensation board).

Notably, each of these 6 highly respected scientific agencies has independently recognized the carcinogenic nature and effect of NDMA and concluded that NDMA should be regarded as and/or anticipated to be a human carcinogen.

NDMA: Background and Properties

NDMA is a semi-volatile organic chemical with the chemical formula $(\text{CH}_3)_2\text{NNO}$ that forms in both industrial and natural processes. It has the appearance of a yellow oil. It is a member of a large class of N-nitrosamines, which includes some of the most potent carcinogens known world-wide. Important sources of external exposure to nitrosamines include tobacco and food products. NDMA has 2 components: the nitroso group (N) and the dimethylamine (DMA). This large class of nitrosamines includes the following that are classified as *probable human carcinogens*: 1) *N*-Methyl-*N*’-nitro-*N*-nitrosoguanidine (MNNG), 2) *N*-Nitrosodi-*n*-butylamine, 3) *N*-Nitrosodiethanolamine, 4) *N*-Nitrosodiethylamine, 5) *N*-Nitrosodimethylamine, 6) *N*-Nitrosodi-*n*-propylamine, 7) *N*-Nitroso-*N*-ethylurea, 8) 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), 9) *N*-Nitroso-*N*-methylurea, 10) *N*-Nitrosomethylvinylamine, 11) *N*-Nitrosornicotine,

12) *N*-Nitrosopyrrolidine, and 13) *N*-Nitrososarcosine are all *reasonably anticipated to be human carcinogens* based on sufficient evidence of carcinogenicity from studies in experimental animals according to the NTP report on carcinogens (14th Edition).

Nitrosamines including NDMA and NDEA play a critical role in the initiation of carcinogenesis^{29,56,57}. N-nitroso compounds including NDMA and NDEA and are used as prototype carcinogens to induce various types of cancer in animal models, including liver, lung, bile duct and pancreatic^{29,58} to test anti-cancer drugs and study the biological mechanisms of cancer causation and cancer pathogenesis. Moreover, extensive studies have demonstrated the cytotoxicity, genotoxicity, carcinogenicity, mutagenicity, as well as reproductive and developmental toxicity of nitrosamines⁵⁹⁻⁶². Nitrosamines have been found to be carcinogenic in over 40 animal species and one or more of the compounds has induced tumors in almost every organ in rodents⁶³. Importantly, NDMA induces cancer via a dose-response⁶⁴, and has demonstrated highly carcinogenic, mutagenic, and teratogenic activity⁶⁵⁻⁶⁷.

Over the past 60 years, NDMA has been extensively studied in experimental animal and human tissue models. Unlike many other carcinogens which require regular administration, ***only a single dose of NDMA is required to cause and initiate cancer*** in multiple animal species, at multiple-sites, and in multiple-strains of both male and female animals. Importantly, NDMA causes cancer in animals even at low doses. NDMA initiates the transformation of normal cells to cancer cells, which is a key characteristic of carcinogens²⁴.

Due to its carcinogenic activity, NDMA is not allowed to be used in manufacturing processes in the United States. It was formerly used in production of liquid rocket fuel, antioxidants, and as additives for lubricants and softeners for copolymers. Potential industrial sources include byproducts from tanneries, pesticide manufacturing plants, rubber and tire

manufacturers, alkylamine manufacture and use sites, fish processing facilities, foundries and dye manufacturers.

NDMA can also be found in many processed foods and beverages such as beer, cured meats, bacon, smoked and salted fish, and cheeses. The estimated daily dietary intake of NDMA in the U.S. population ranges from 0.03 to 0.06 $\mu\text{g}/\text{day}$, depending on age, with adults aged 20–49 years experiencing exposure of 0.06 $\mu\text{g}/\text{day}$ (or 0.08 $\mu\text{g}/\text{day}$ when beer is included)⁶⁸. NDMA is also an unintended byproduct of the chlorination of wastewater and drinking water at treatment plants that use chloramines for disinfection⁶⁹. For example, water-resource managers in California became concerned about the potential for NDMA to reach ground water after NDMA was detected in two drinking-water supply wells located near a water reclamation facility where treated wastewater was injected directly into the subsurface to recharge ground water. Two drinking water supply wells in Orange County, California, were removed from service in the year 2000 following the detection of NDMA at concentrations of 30–40 ng/L (nanograms per liter), which were above the California Department of Health Services' drinking-water action level of 10 ng/L for NDMA.

Nitrosamines including NDMA have caused cancer in every animal model tested. Specifically, NDMA and other nitrosamines have caused cancer in multiple organs in at least 40 animal species (male and female) belonging to 36 genera, 25 families, 17 orders, and 5 classes, including higher primates⁷⁰. ***No animal species has been found to be resistant***⁷⁰. Importantly, many scientific studies have elegantly demonstrated that NDMA plays a critical role in the initial formation of cancer^{29,56,57}. Since NDMA reliably and reproducibly causes cancer, it is used routinely world-wide by many scientists as a gold-standard to initiate tumor growth in the laboratory, including liver, esophagus, bladder, and bile duct tumors in animals, to test anti-cancer drugs and study the biological mechanisms of the initiation and promotion of cancer²⁹. NDMA is

used in my laboratory to initiate tumor growth in animals and study mechanisms of cancer causation in murine and human tissues as well as cells. The commonly used colon-specific carcinogen azoxymethane (AOM) is an isomer of NDMA and is used world-wide as a gold-standard to induce experimental colitis-associated colon cancer along with dextran sodium sulfate (DSS)⁷¹⁻⁷³.

NDMA is a Tumor Initiator and Tumor Promoter

NDMA is a complete carcinogen that functions as both a tumor initiator and tumor promoter. It initiates cancer in various animal models without the administration of other carcinogens and also promotes the rapid growth of existing cancer. While the classic model of initiation-promotion-progression of cancer has helped scientists explain their observations in many animal cancer models, the idea that cancer is a *cell-autonomous* disease, where only the tumor cell alone is viewed as important, is outdated. The simple notion that cancer is a tumor cell disease driven by mutation/genetic changes and selection for fast growing and increasingly malignant cell clones has gradually yielded to a more integrated view that cancer requires the support from the “tissue microenvironment” in the “tumor bed” (Figure 1). It is now established that the tumor microenvironment is comprised of a variety of cells, including various inflammatory cells such as tumor-associated macrophages, that are critical for tumor growth, and angiogenesis^{1,33}.

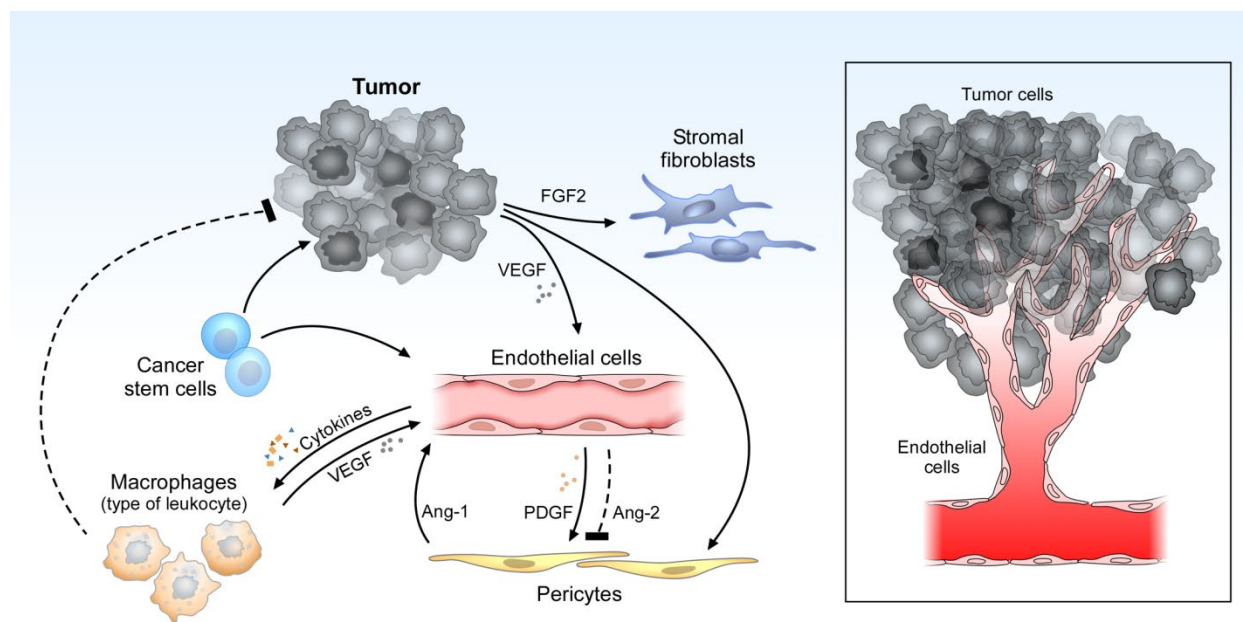


Figure 1. The tumor microenvironment includes non-tumor cells, such as endothelial cells and pericytes (making up tumor blood vessels), inflammatory cells (including macrophages), fibroblasts, and cancer stem cells. These cells stimulate the production of cancer stimulating cytokines/growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2). (Created by Steve Moskowitz, Advanced Medical Graphics).

The distinction between tumor “initiator” and “promoter” has become scientifically less significant and relevant. In fact, instead of viewing carcinogens as simply “genotoxic vs nongenotoxic”, there are 10 key characteristics of carcinogens that describe the different mechanisms of how a chemical can cause cancer^{24,25}. Thus, we have a more complex picture of cancer: cancer stem cells (or tumor-initiating cells), circulating tumor cells, and stromal processes such as inflammation, angiogenesis, oxidative stress, immunosuppression and cell death are critical for tumor initiation, tumor promotion, tumor dormancy escape and tumor progression. NDMA stimulates tumor progression via actions directly on the tumor cells and through the tumor microenvironment such as triggering oxidative stress, inflammation, immunosuppression, and angiogenesis.

Low Dose NDMA Causes Many Cancer Types in Multiple Species

NDMA has consistently shown potent carcinogenicity in all laboratory animal studies. Common sites of NDMA-induced cancerous tumors observed in extensive animal studies include liver, bile duct, lung, stomach, intestine/colon, kidney, bladder, brain, gallbladder, sarcomas, hemangiomas (blood vessel tumors), testicular, blood (e.g., lymphoma and leukemia), head and neck (e.g., nasal) and pancreatic cancer types.

In Peto *et al.*, NDMA caused malignant tumors of the liver, bile duct, and gall bladder at doses of 1 to 5 ppm in rats and mice^{19,74}. The number of NDMA-induced liver neoplasms was directly proportional to the dose, with no indication of a threshold¹⁹. An increased incidence of NDMA-induced hepatic tumors was observed at doses as low as approximately 0.1 mg/kg body weight per day in the rats¹⁹. In a high profile publication in the journal *Nature*, Terracini and Magee in 1964 demonstrated that a single dose of NDMA caused kidney tumors and hepatocellular carcinoma in animals⁷⁵. Moreover, 5 mg/L of NDMA in tank water induced hepatocellular carcinomas, adenomas and blood tumors (hematopoietic system) in frogs⁷⁶.

In Arai *et al.* (1979), oral administration of NDMA (1 ppm) in rats caused liver tumors (e.g., hepatocellular carcinomas and hemangioendotheliomas), adrenal adenomas, pituitary adenomas, interstitial cell tumors of the testis, ovarian tumors, and leukemia⁷⁷. In Moiseev and Benemanski *et al.*, NDMA at 1.1 ppm induced lung, liver, and kidney cancers in rats and mice⁷⁸. In Klein *et al.*, NDMA at 0.2 to 1 ppm via chronic inhalation induced nasal tumors, squamous cell carcinoma, and sarcomas in rats^{79,80}. In Otsuka and Kuwahara *et al.* (1971), administration of NDMA via diet or subcutaneous injection for 16 to 92 days induced lung adenomas and carcinomas, benign and malignant hemangiomas of the liver and soft tissue, kidney, spleen, and blood tumors (e.g., leukemia) in mice⁸¹.

Kuwahara et al. (1972) demonstrated that weekly subcutaneous or intraperitoneal injections of NDMA to various strains of mice for 1 to 25 weeks resulted in very high incidences of tumors in multiple organs and tissues including liver (hemangioendothelial sarcomas), soft tissues, lung tumors⁸². Endothelial cells (blood vessels) of various organs and tissues were important targets of NDMA⁸². Remarkably, exposure to NDMA (25 µg as a 0.05% solution in drinking water) caused kidney tumors (e.g. adenomas) after only 7 days⁸³. In studies by Terracini et al. (1966), NDMA induced liver vascular tumors, hepatomas, lung adenomas, and kidney adenomas⁸³. A no-effect level of NDMA could not be established in the studies by Terracini et al. (1967), as all doses via dietary NDMA caused liver cancer in the rats (ranging from 2 to 50 ppm)⁸⁴. Administration of NDMA in the drinking water (1 ppm) caused stomach cancer⁸⁵. Tomatis et al. (1967) showed that NDMA (1 mg/animal over 5 weeks) or a single dose of NDMA to Syrian golden hamsters induced cholangioadenomas, cholangiocarcinomas (bile duct cancers), and hemangiosarcomas as well as hemangioendotheliomas of the liver⁸⁶. In Campbell et al. (1974), injections of 10, 20, or 30 mg/rat at day 21 or 70 after birth produced 38% kidney tumors after 286-369 days (41% of kidney tumors were of the renal cell type and 59% were stromal nephromas); whereas no control rats developed kidney tumors⁸⁷.

Doses of NDMA required for tumor induction are low (e.g., 10 µg/kg in the food for mice and 33 µg/kg in the food for rats)¹⁹. In Peto et al., the lowest dose of NDMA (0.25 ppm) induced liver tumors, which were non-existent or less in the control (unexposed to NDMA)¹⁹. In contrast to animal cancer studies with NDMA, a “no-effect level” of NDMA of < 1-2 ppm in the food may be extrapolated based on animal studies⁸⁸. For the nitrosamine class including NDMA and NDEA, the maximal allowable concentrations as established by the FDA are absolutely necessary⁸⁸.

There has been clear, consistent evidence of NDMA causing cancer in studies in a wide variety of animal species via every type of administration including oral ingestion, inhalation, intratracheal instillation, intraperitoneal, and subcutaneous administration.

NDMA: Oral vs. Inhalation

NDMA is a potent carcinogen regardless of the route of administration. NDMA has been evaluated in numerous animal bioassays using various routes of exposure and experimental protocols. Significant increases in tumors were observed in male and female, mature and immature animals and in essentially all species that were evaluated (e.g., mice, rats, hamsters, rabbits, and guinea pigs). Carcinogenic activity was observed following both oral and inhalation exposure to NDMA as well as following intraperitoneal or subcutaneous injection of animals. NDMA causes benign and malignant tumors following administration by various routes, including ingestion and inhalation, in a wide variety of organs, including the liver, kidneys and respiratory tract. Absorption of NDMA mainly occurs in the respiratory and digestive tracts, sometimes in the dermal tracts. Tumors were observed in all of the following studies: at least five studies in rodents (all in rats) exposed to NDMA orally, four studies in rodents (3 in rats and 1 in mice) exposed to NDMA by inhalation and one study in hamsters exposed to NDMA intratracheally.

*Studies of animals chronically exposed to NDMA via **oral administration***

In 1956, Magee and Barnes first reported the induction of malignant liver tumors in rats given oral NDMA in their diet⁸⁹. In 1962, Magee and Barnes observed a high incidence of kidney tumors in rats given oral NDMA in their diet for 1-4 weeks⁹⁰. In these studies, a single oral dose of NDMA also increased the number of kidney tumors in rats compared to control rats⁹⁰. NDMA given orally via the drinking water or the diet also causes liver tumors (e.g., hepatocellular carcinomas and hemangioendotheliomas), adrenal adenomas, pituitary adenomas, interstitial cell

tumors of the testis, and ovarian tumors in rats⁷⁷. In Le Page et al. (1969), rabbits given NDMA in their diet (25 to 50 ppm) developed hepatocellular (liver) tumors that spread to the lungs and kidney as well as benign papillary cholangiomas in 17-60 weeks⁹¹. In these studies, the histological appearance of the tumors was similar to the tumors caused by NDMA in rats or guinea-pigs^{91,92}. Guinea-pigs given 25 or 50 mg NDMA/kg in their diet for 6-49 weeks developed papillary cholangiomas and liver cell carcinomas⁹².

In Peto et al. (1991), groups of 60 male and female Colworth rats were exposed for their entire life (average median survival of approximately 2.5 years) to one of 15 different doses of NDMA (administered in water at concentrations ranging between 0.033 to 16.896 ppm NDMA). The number of tumors observed was compared to that observed in the control group of 120 male and female rats. These studies included a total of 4,080 rats. This experimental study involved multiple doses with several animals per dose, and all cancer types were analyzed taking care to reflect rates of cancers observed and expected in the exposed group. The follow-up of rats was performed until natural death occurred, which in some cases was over more than 3.5 years. Note that in experimental cancer studies, rats are generally sacrificed after 2 years of life. This results in not being able to observe the natural increased rate of death from cancer in the last months of life. However, like the age dependent cancers seen in humans, this rate increases very rapidly in animals at the end of their life. Thus, Peto et al. concluded that in rats exposed to low levels of NDMA starting at 6 weeks of age, mortality due to liver cancer would be approximately 7-fold greater in the animals allowed to succumb naturally than in those exposed to NDMA and then monitored for only 2 years¹⁹. The survival rate of animals exposed to NDMA decreased as the dose of NDMA increased.

Lijinsky *et al.* (1984) demonstrated that NDMA in the drinking water caused a large incidence of liver cancers (e.g., 35% hemangiosarcomas, as well as hepatocellular tumors) in rats⁹³. Even the low NDMA dose caused liver tumors in half of the rats⁹³. In studies by Lijinsky *et al.* (1981), at higher doses, NDMA caused earlier death in the rats, specifically almost exclusively hemangiosarcomas of the liver⁹⁴. Lijinsky *et al.* (1987) observed that NDMA induced a high incidence of mesenchymal tumors of the kidney and alveolar-bronchiolar tumors of the lungs as well as liver tumors in rats with 6 mg NDMA/kg twice weekly for 30 weeks⁹⁵. Tumors developed in the liver, lung, and kidney^{77,93-95}. In some cases, such as Terracini *et al.* (1966), the period of exposure to NDMA was relatively short, as a week of NDMA in the drinking water was sufficient to cause tumors in the kidney and lungs⁸³. In Terracini *et al.* (1967), a dose-response study was conducted in rats in which NDMA in oil solution was added to the diet. After 120 weeks, liver tumors were observed in rats⁸⁴.

In rodents, liver tumors are most commonly induced when NDMA passes through the liver at relatively low concentrations, such as when the compound is absorbed at low concentrations from the GI tract. At greater concentrations, as when NDMA is given via pulsed doses by gavage (force-fed), much of the dose escapes the liver's first pass metabolism and reaches other organs in sufficient quantities to induce tumors through the rest of the body⁹⁵.

Clapp *et al.* (1968) observed that NDMA given orally in the drinking water induced liver and lung tumors in 97% and 99% of mice, respectively, compared to 4% and 39% of controls (not exposed to NDMA) in mice⁹⁶. Liver tumors were mainly hemangiosarcomas and hemangioendotheliomas⁹⁶. Clapp and Toya (1970) observed oral NDMA via drinking water induced nearly 100% of lung adenomas (increase over controls by a factor of 2) and 96% of liver hemangiosarcomas in mice⁹⁷. Clapp *et al.* (1971) also reported lung adenomas and liver

hemangiosarcomas in two strains of BALB/c and RF mice from exposure to NDMA in their drinking water⁹⁸. Otsuka and Kuwahara et al. (1971) demonstrated that daily administration of NDMA in the diet of mice for 16 to 92 days induced lung adenomas and carcinomas, benign and malignant hemangiomas of the liver, hemangioendothelial sarcomas, soft tissue tumors, and/or kidney tumors⁸¹.

*Studies of cancer in animals chronically exposed to NDMA via **inhalation***

The carcinogenicity of inhaled NDMA has been evaluated in several studies by Drukrey et al. (1967), Moiseev and Benemanski (1975), and Klein et al. (1991). In studies by Drukrey et al. (1967), twice weekly 30-minute exposures to NDMA by inhalation (vapor) produced malignant nasal cavity tumors in 8 of 12 rats⁹⁹. Moiseev and Benemanski et al. (1975) showed that rats and mice continuously exposed to 0.07 ppm NDMA for 25 and 17 months, respectively, developed significantly increased lung, liver, and kidney tumors^{78,79}. Tumor types included various carcinomas and sarcomas in the lung, liver, and kidneys, and hemangiomas in the liver. Increased incidences of nasal, liver, lung, and kidney tumors were observed in rats exposed to NDMA inhalation. Since the tumors associated with exposure to 0.07 ppm NDMA are consistent with those produced by NDMA in oral and injection studies, 0.07 ppm is considered to be the chronic exposure level (CEL) for rats and mice for continuous inhalation over time.

In Moiseev and Benemanski et al. (1975- study 1), the effects of NDMA inhalation exposure on male and female Wistar rats were evaluated with a group of 87 rats exposed for 25 months to 50 µg NDMA/kg body weight/day, a group of 61 rats exposed to 200 µg NDMA/kg body weight/day, and a group of 77 control rats unexposed to NDMA. Exposure to 1.1 ppm in rats resulted in increased incidence of lung cancer: exposed group (12/61) vs. control group (5/77); liver cancer: exposed group (12/61) vs. control group (3/77), and kidney cancer: exposed group

(32/61) vs. control group (2/77)⁷⁸. In Moiseev and Benemanski et al. (1975- study 2), a group of 101 mice exposed to 200 µg NDMA/kg body weight/day (1.1 ppm) via inhalation were compared with a group of 81 unexposed control mice. NDMA induced lung cancer: exposed group (19/101) vs. control group (3/81); liver cancer: exposed group (6/101) vs. control group (0/81); and kidney cancer: exposed group (4/101) vs. control group (0/81)⁷⁸.

In the Klein et al. studies from 1989 to 1991, inhalation of low dose (0.2 ppm) NDMA caused a remarkable 86% nasal tumor incidence in rats⁸⁰. Tumors occurred mainly in the nasal cavity, with the highest incidences in the groups receiving 1.0 and 0.2 ppm NDMA (19/36 and 31/36 tumor-bearing animals, respectively). No nasal or respiratory tract tumors were observed in the control animals. At 1 ppm NDMA, 47% of the tumors were aesthesioneuroblastomas, whereas following inhalation of 0.2 and 0.04 ppm NDMA observation of this tumor type was reduced to 6% to 15%, respectively. In the 0.2 and 0.04 ppm groups, mucoepidermoid tumors represented the greatest proportion of tumors. The studies are important as they confirm that NDMA is a potent carcinogen via inhalation. Here, the rats were allowed to live until death before performing a necropsy. Some rats were bearing more than one tumor evidencing that NDMA increases the incidence of nasal, liver, lung, and kidney tumors^{78,79}.

Thus, NDMA causes cancer in studies of a wide variety of animal species via multiple types of administration including oral ingestion and inhalation. The doses that are absorbed into the body whether orally ingested or inhaled are equally potent.

THE TEN KEY CHARACTERISTICS OF CARCINOGENS

Over the past decade, it has become clear that mechanistic studies are very important in determining whether a chemical causes cancer^{24,25,55}. Prior mechanistic studies focused on “genotoxic” vs. “non-genotoxic” mechanisms of carcinogens, however, this is an oversimplification. When the IARC updated the classification of carcinogens during its 2012 Working group meeting, two unmet needs were realized: (1) there was no broadly accepted method for identifying, organizing, searching, and summarizing mechanistic data when deciding whether a chemical is a carcinogen; and (2) human carcinogens commonly show one or more of 10 key properties or characteristics which lead to cancer via multiple mechanisms²⁴. These 10 key characteristics are different from the classic hallmarks of cancer which describe the properties of tumor cells and neoplasms compared to normal cells set forth by Hanahan and Weinberg^{100,101}. Originally, six hallmarks were described in 2000 along with two enabling characteristics (genome instability and inflammation), and two emerging hallmarks (deregulated metabolism and immune system evasion) were added in 2011. However, the hallmarks of cancer do not describe the properties of chemicals or agents that cause cancer²⁴. Thus, while the hallmarks are the properties of cancer cells, the key characteristics are the properties of human carcinogens that induce cancer. During two 2012 workshops, organized by IARC in Lyon, France, on Tumor Site Concordance and Mechanisms of Carcinogenesis, the participants extensively debated the mechanisms by which agents identified as human carcinogens cause cancer. These cancer experts noted that human carcinogens, while operating individually through distinct mechanisms, often share one or more characteristics related to the multiple mechanisms by which agents cause cancer^{24,25}. This led to the identification of 10 key characteristics (KCs) of human carcinogens^{24,25}. Since the biologically plausible mechanisms of action of carcinogens are key to identifying carcinogens, the key

characteristics of carcinogens were developed to provide a broad view of the properties of human carcinogens leading to cancer²⁴.

The Use of Key Characteristics in the Identification of a Carcinogen

Mechanistic studies are key to understand whether a chemical causes cancer and understanding the human relevance of findings in experimental animal studies. For example, mechanistic evidence can add biological plausibility to epidemiological findings, thereby strengthening causal association between a chemical and cancer²⁵. Biological plausibility is the proposal of a causal association (e.g., a relationship between a cause and an outcome) that is consistent with existing biological and medical knowledge. In this case, the question, is NDMA or NDEA a human carcinogen? Strong mechanistic data can play a pivotal role in the overall carcinogen hazard classification in which human epidemiologic studies are limited⁵⁵.

These 10 key characteristics help identify the mechanisms contributing to the induction of the observed animal tumors from carcinogens and determine whether analogous mechanisms may be operative in humans. If similar mechanisms in animals and humans occur, this provides strong support for cancer causation of a chemical. If the key characteristics are present, they help to create the necessary tumor microenvironment for tumor initiation and progression. The IARC has applied the key characteristics in mechanistic data evaluations of more than 50 diverse chemicals and complex exposures since 2015⁵⁵ and have included them into the January 2019 Preamble of the IARC Monographs. Prior to the introduction of the key characteristics approach, there was no widely accepted method to systematically search for and organize relevant mechanistic evidence²⁵. Other authoritative bodies, including the IARC, NTP (e.g., Report on Carcinogens), EPA, CalEPA, and OEHHA are increasingly using the key characteristics to identify carcinogens and better understand the mechanisms underlying how chemicals cause cancer.

These 10 key characteristics of human carcinogens provide the basis for an objective approach to identify and categorize scientific findings relevant to cancer mechanisms when assessing whether a chemical is a potential human carcinogen^{24,25}. This systematic approach will assist future IARC working groups and other agencies in evaluating chemicals and pharmaceutical agents as potential human carcinogens, especially in the absence of convincing epidemiological data on many human cancers²⁴.

The key characteristics of carcinogens described by Smith et al. (2016)^{24,25} which are utilized by IARC are as follows:

10 Key Characteristics of Carcinogens

1. Can the agent act as an electrophile or be metabolically activated to an electrophile?
2. Is the agent genotoxic?
3. Does the agent alter DNA repair or cause genomic instability?
4. Does the agent induce epigenetic alterations?
5. Does the agent induce oxidative stress?
6. Does the agent induce chronic inflammation?
7. Is the agent immunosuppressive?
8. Does the agent modulate receptor-mediated effects?
9. Does the agent cause immortalization?
10. Does the agent alter cell proliferation, cell death, or nutrient supply?

The first four characteristics focus on the genotoxic nature of the chemical agent. The last six characteristics focus on the nongenotoxic mechanisms in the tumor microenvironment. These 10 key characteristics of human carcinogens are a roadmap for identifying and categorizing

scientific findings relevant to cancer mechanisms when asking whether a chemical or agent is a potential human carcinogen²⁴. The 10 key characteristics help in the identification of carcinogens as a first step in cancer prevention to prevent human suffering and death from cancer²⁵. I used these key characteristics to determine the biological plausibility of the mechanisms of action for both NDMA- and NDEA-induced carcinogenesis. The range of evidence available to determine whether NDMA exhibits these 10 key characteristics includes the following: animal cancer bioassays, studies of specific biological mechanisms in tissues and cells derived from humans, studies of specific biological mechanisms in tissues and cells derived from animals, and studies from exposures to humans. Starting in 1956, scientific studies show that NDMA has been extensively characterized as a presumed human carcinogen for decades. There is little available data from human exposures aside from incidental exposures because of occupation or diet, and unfortunate incidents of intentional poisoning. My analysis as to whether NDMA has each of these key characteristics is below.

NDMA EXHIBITS 9 OUT OF THE 10 KEY CHARACTERISTICS OF
CARCINOGENS

Most human carcinogens exhibit more than 1 of the 10 key characteristics with known carcinogens having an average of 3 to 4 key characteristics (e.g., benzene and polychlorinated biphenyls (PCBs)²⁴. By stunning contrast, NDMA exhibits 9 out of the 10 key characteristics which makes NDMA an extremely potent carcinogen with multiple mechanisms of action for its activity as a human carcinogen. My analysis below determined whether NDMA exhibits each of these key characteristics of carcinogens.

9 Key characteristics of NDMA

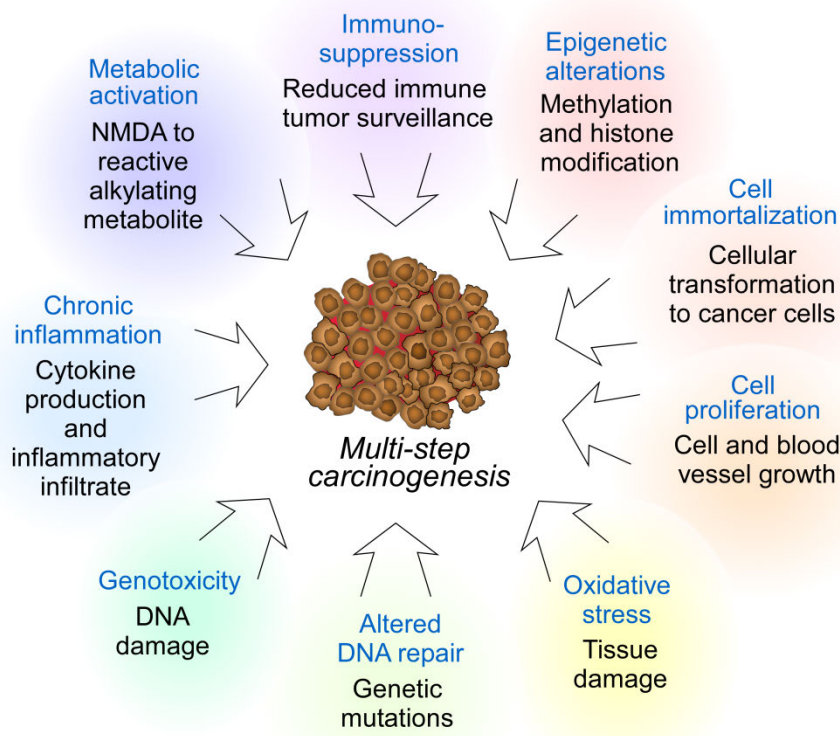


Figure 2. NDMA exhibits 9 of the 10 key characteristics of carcinogens.

Key Characteristic #1: NDMA is Metabolically Activated to Electrophiles Inducing the Formation of DNA Adducts

General Description

Many carcinogens do not directly cause cancer as the “parent molecule” but instead must first undergo a transformation process called metabolic activation to induce cancer. *Metabolic activation* is defined as the chemical conversion of a relatively benign (“harmless”) substance into a more hazardous (“cancer causing”) substance by biochemical processes in the cells and tissues. It is now well established with many thorough scientific studies that the cancer causing activity of

NDMA results from its metabolic transformation within susceptible tissues in the body to a chemically reactive agent which methylates a variety of molecules such as DNA forming DNA adducts¹⁰². Methylation is a chemical reaction in which a methyl group replaces a hydrogen atom.

Thus, before NDMA can cause cancer, the initial NDMA molecule that is ingested via a contaminated tablet or absorbed must undergo bioactivation to electron-seeking molecules (called electrophiles) that bind to DNA to form addition products, referred to as adducts, which lead to DNA damage and mutations. DNA adducts are segments of DNA bound to a cancer-causing chemical. The formation of DNA adducts is a key process that initiates the transition to a cancerous cell from a normal cell (carcinogenesis). The ability to form DNA and protein adducts is a common property of electrophilic and metabolically activated human carcinogens. For example, the classic mechanism of smoking includes metabolic activation of tobacco smoke to DNA adducts¹⁰³. The measurement of DNA adducts is one of the most common methods of assessing electrophilic activity both *in vitro* (outside the body) and *in vivo* (inside the body) to determine if this key characteristic occurs. The DNA adduct O6-alkylguanine has been shown to alter the DNA resulting in a mutation. The ability of carcinogens to form DNA adducts can lead to cancer¹⁰².

Why is metabolic activation important to the mechanism of carcinogenesis?

While some chemical carcinogens can directly cause cancer, many other carcinogens must undergo biotransformation by enzymes via metabolic activation before the chemical can cause cancer¹⁰⁴. Metabolism to an active metabolite is required for NDMA-induced cancer as the carcinogenic effects of NDMA are due to a metabolite rather than the compound itself. Metabolic activation of NDMA is required for its genotoxic and cancer causing activity. Several human enzymes can bio transform relatively inert chemical compounds to potent toxic and carcinogenic metabolites or reactive intermediates. These enzymes include cytochrome P450s (CYPs)¹⁰³. CYPs

are a superfamily of enzymes that catalyze chemical reactions of a wide variety of compounds, including drugs, carcinogens, and other chemicals. Many CYPs also activate carcinogens to electrophilic (electron loving), highly reactive compounds that bind to DNA which initiates the process of cancer formation¹⁰⁵.

Electrophiles and their nucleophilic targets can be described by their strength, which can predict how strong a reaction they undergo to cause cancer²⁵. Electrophiles are very dangerous since they have electron withdrawing groups capable of binding to N and O sites in the DNA which generates DNA adducts leading to cancer²⁵. Thus, any tissue or cell in the human body that expresses the enzyme (e.g., CYP) can metabolically activate the carcinogen. The cytochrome P450 enzymes that induce the metabolic activation of NDMA are present in many human tissues and cells including blood, liver, small and large intestine, bladder, lung, kidney, prostate, and pancreas¹⁰⁶.

NDMA induces metabolic activation in animal tissues and cells

Extensive studies in animal carcinogenesis bioassays, animal tissues and cells demonstrate that NDMA can cause cancer via metabolic activation inducing DNA adducts. NDMA is an alkylating agent that causes cancer via molecules called electrophiles. The electrophiles generated by NDMA induce DNA adducts⁶³. NDMA is capable of methylating many cell types to generate DNA adducts to initiate cancer throughout the body. In a high profile scientific paper in the high impact journal *Nature*, NDMA was elegantly demonstrated to cause persistent levels of DNA adducts (e.g. 6-methylguanine) in the rat liver and kidney DNA, which lead to cancer¹⁰². Liver microsomes from NDMA-treated rats also show an increased ability to convert NDMA to mutagenic molecules that can cause cancer.

While NDMA-induced DNA adduct formation occurs at a number of DNA sites, certain sites are preferred. The principal DNA adduct formed following exposure to NDMA is *N*7-methylguanine (representing about 65% of all adducts formed initially upon exposure); *O*6-methylguanine (O6-meG) is a secondary adduct (representing about 7% of all adducts formed initially upon exposure). Thus, NDMA methylates the DNA at certain sites to cause cancer in many tissues remote from the site of administration¹⁰⁷. NDMA at low doses (e.g., 0.2 to 2.6 ppm) in the drinking water of rats can induce DNA adducts (e.g., O6-meG) in the blood leukocytes (white blood cells) and liver leading to cancer formation^{63,108}. NDMA-induced DNA adducts accumulate rapidly in the blood leukocytes and liver of rats, reaching a steady state within 2-7 days of NDMA exposure¹⁰⁸. Importantly, the steady state DNA adduct levels were approximately linear related to NDMA dose-rate¹⁰⁸. NDMA-induced adducts are harmful as they cause mutations and stimulate unabated cell proliferation⁶³.

NDMA-induced tumors typically occur in tissues that have the ability to metabolize NDMA. The occurrence of tumors is related to the levels of cytochrome P450 (principally CYP2E1) within the cell and the ability of cells to metabolize NDMA by the alpha hydroxylation pathway. The cancer-causing activity of NDMA is directly dependent upon the cytochrome P450 (e.g., CYP2E1)-dependent metabolic conversion of NDMA to highly reactive molecules such as the methyldiazonium ion^{109,110}. In rat studies NDMA is shown to be metabolized primarily in the liver by an enzyme called cytochrome P450 2E1 (CYP2E1) to a methyldiazonium ion¹¹⁸. CYP2E1 is responsible for at least 60% of the NDMA-induced DNA methylation in rat hepatocytes¹¹¹. The cytochrome P-450 mixed-function oxidase system also metabolizes NDMA to a mutagen¹¹². Activation of NDMA to a mutagen is cytochrome P450 dependent¹¹². Importantly, mutations can lead to cancer. Thus, NDMA can induce tumors in any cells which express cytochrome P450

enzymes¹¹². NDMA is rapidly metabolized in any tissue that expresses the cytochrome P450 enzymes and can initiate cancer in that tissue. In addition, NDMA can act as a tumor-promoter to other sites in the body via the key characteristics and stimulate human cancer via inflammation, oxidative stress, immunosuppression, proliferation, cell death and angiogenesis (the growth of new blood vessels). Thus, NDMA can stimulate many cancer types including colorectal/intestinal, esophageal/pharyngeal, gastric, kidney, liver, lung, pancreatic, prostate, bladder and blood (e.g., lymphoma, leukemia and multiple myeloma) via metabolic activation, inflammation, angiogenesis, and the other key characteristics of carcinogens.

Even at low doses in the rat's drinking water, NDMA stimulated the formation of DNA adducts (e.g., O6-methylguanine) in the white blood cells (called leukocytes) and the liver within 2 to 7 days exposure¹⁷². Blood leukocytes are a reliable surrogate tissue for monitoring the biologically significant exposure to NDMA. NDMA causes DNA adduct formation and gene mutations (e.g., G:C to A:T) in various tissues such as the liver. NDMA-induced formation of O6-methylguanine in DNA is important in tumor initiation caused by NDMA. The formation of the DNA adduct N7-methylguanine is proportional to NDMA dose in the liver and kidney in rats orally administered NDMA. O6-methylguanine DNA adducts that accumulated over 28 days were linearly related to NDMA dose in the rat liver. The administration of NDMA in drinking water to rats also resulted in a dose-dependent increase of N7-methylguanine adducts in the liver at 28 days.

The probable human carcinogen formaldehyde can be formed from NDMA via chemical reactions (e.g., oxidation) with liver microsomes in rats, mice and hamsters^{112,113}. Additionally, the formaldehyde and acetaldehyde produced in the metabolism of NDMA are carcinogens. After NDMA treatment *in vivo* in animals or in tissue slices *in vitro*¹¹⁴⁻¹¹⁶, the major product was 7-methylguanine. In rats, even a single oral NDMA dose induces O6-methylguanine DNA adducts

in the kidney which parallels earlier findings in which oral or intraperitoneal administration of NDMA to rats increases the incidence of kidney tumors^{90,117}. Changes in DNA (e.g., G:C to A:T transitions) have been observed in the *ras* oncogene in mouse lung tumors induced by NDMA¹¹⁸. DNA methylation was studied in peripheral blood lymphocytes (PBLs) collected from Sprague-Dawley rats exposed to a single dose of NDMA¹¹⁹. The O6-meG-DNA adduct formation in PBLs and hepatocytes, at 2-24 h following the exposure to NDMA, was analogous for both types of cells¹¹⁹. In another study, DNA was extracted from livers, kidneys and lungs of Syrian golden hamsters at various times (up to 96h) after injection of a cancer causing dose of [14C]NDMA¹²⁰. At 7h after NDMA administration liver DNA was alkylated to the greatest extent, followed by that of the lung and kidney, O6-methylguanine was the most persistent alkylated purine in the hamster tissues¹²⁰.

DNA adducts are also generated by NDMA in large animals. In monkeys, orally dosed with 0.1 mg NDMA/kg body weight, the DNA adduct O6-methylguanine was detected in 32 tissues examined¹²¹. When monkeys were exposed to 0.1 mg/kg oral NDMA, the DNA adduct O6-methylguanine was detected in all tissues studied including stomach, esophagus, large intestine, small intestine, pancreas, liver, blood (white blood cells), ovary, bladder, spleen, brain (e.g. cerebellum, cerebrum, and brain stem), uterus, bone marrow, lymph nodes, prostate, adrenal, pituitary, skeletal muscle, kidney, heart muscle, heart blood vessels, skin, and lung¹²¹. The highest levels were in the gastric mucosa and liver with elevated levels also present in white blood cells which circulate throughout the body, the esophagus, ovaries, pancreas, bladder and uterus¹²¹. Thus, NDMA can cause cancer in all these tissues. Humans share over 90% of their DNA with their primate cousins the monkeys and chimpanzees¹²² and as one of our closest living evolutionary relatives, non-human primates (e.g. monkeys) are especially suited to teach us about ourselves.

The monkey species is widely used for biomedical research due to its high sequence similarity with humans (>93% for protein-coding genes)¹²³. Thus, monkeys provide meaningful scientific evidence on cancer formation mechanisms that applies to humans⁵⁰. NDMA also has been demonstrated to induce DNA adducts in various *human tissues* including liver, lung, bladder, colorectal/intestinal, pancreas, esophagus, buccal mucosa (the lining of the cheek), and placenta (an organ that develops in the uterus during a pregnancy)¹²⁴⁻¹³⁰. Thus, NDMA exposure to humans can form similar adducts throughout the human body to initiate cancer including these and other tissues including gastric, kidney, and blood.

In large animals (e.g., primates), low doses of NDMA given systemically caused the formation of DNA adducts (e.g., O6 methylguanine DNA adducts) in the esophageal tissue of oral mucosa. In monkeys, NDMA induced O6-methylguanine DNA adducts four hours post-administration in various tissues (e.g., kidney, esophagus, stomach, brain) similar to what was observed in the liver. This is unlike what is typically observed in rodents, in which adducts occurred primarily in the liver. To test if the fetus in primates is sensitive to the formation of cancer initiation-related DNA adducts, pregnant patas monkeys were given 1.0 or 0.1 mg/kg *NDMA*. Elevated levels of O6-methylguanine were detected in fetal liver, lung, kidney, spleen, brain, and placenta in a study in which pregnant patas monkeys were administered a single gastric NDMA dose of 1 mg/kg body weight (bw)¹³¹.

Human tissue and cells

This first key characteristic of NDMA was demonstrated in a human by the unfortunate case of poisoning, which was reported by Herron and Shank (1980)¹²⁶. A considerable amount of DNA methylation (O6-methylguanine and 7-methylguanine) was found in the victim's liver which evidenced that NDMA is metabolically activated in humans¹²⁶. DNA molecules in liver cells from

humans exposed to NDMA contain the methylated purines N7-methylguanine and O6-methylguanine, indicating alterations in DNA by NDMA. Human tissue (e.g. breast xenografts) also metabolize NDMA to active intermediates that react with DNA¹³².

This mode of action, and a crucial role of O-6methylguanine DNA-methyltransferase, is similar in rodents and humans (WHO, 2002). O-6methylguanine is the most potent premutagenic lesion induced by NDMA. Many tissues and cells in animals and humans metabolize NDMA including stomach, esophageal, pancreatic, bladder, liver, kidney, lung, prostate and leukocytes (white blood cells). In fact, in human lymphocytes, the identical DNA adducts as those observed in experimental animal studies are detected. In addition, adducts have been detected in human liver DNA following NDMA exposure¹²⁶. Thus, humans, like rodents, activate NDMA metabolically in an identical fashion as laboratory animals.

Importantly, metabolism of NDMA is similar in animals (e.g., rats) and humans, mediated by the identical enzyme, CYP2E1. NDMA metabolism in animals and humans is virtually identical, allowing scientists to conclude that NDMA is a human carcinogen. The mechanism of action of NDMA is related to metabolism by cytochrome P-450 enzymes to generate methyldiazonium ions and subsequent DNA adducts, predominantly N7-methylguanine and O6-methylguanine.

Conclusion – Key Characteristic #1

As cited above, there is overwhelming evidence that NDMA is metabolically activated via the formation of DNA adducts to cause cancer. The formation of DNA adducts such as O6-methylguanine is of critical importance in the cancer-causing activity of NDMA. Importantly, there is also convincing evidence that the biological activity of NDMA in humans does not differ to any meaningful degree from that in experimental animals and there is a human study that

confirmed a considerable amount of DNA methylation (O6-methylguanine and 7-methylguanine) in the victim's liver¹²⁶. As such, we can predict with a high degree of confidence that NDMA is carcinogenic in humans. Thus, NDMA requires metabolic activation to exert its cancer causing activity.

It is biologically plausible that NDMA causes cancer via key characteristic #1.

Carcinogen Key Characteristic #2: NDMA is Genotoxic

General Description

Genotoxicity is defined as the capability of a chemical to cause DNA damage, alter the genome (mutation), or both²⁵. Mutagens are agents that damage DNA and can, depending on the ability of an organism to repair DNA damage, lead to permanent changes (mutations) in the DNA sequence. Clastogenic is a mutagen that results in sections of chromosomes being deleted, added, or rearranged. This process is a form of mutagenesis which can lead to cancer. The term “genotoxic” refers to an agent that induces DNA damage, but this damage may or may not necessarily be processed by the cell into a mutation. If an agent is found to induce DNA damage, it is a genotoxin, and if it is shown that the agent also induces mutations in a mutagenicity assay, it can be classified as a mutagen.

Why is genotoxicity important to the mechanism of carcinogenesis?

The link between genotoxicity and cancer causation is well-established as cause and effect and has shaped standardized testing of carcinogens for decades¹³³. Genotoxicity can arise from DNA strand breaks, DNA adducts, DNA-DNA crosslinks, and DNA-protein crosslinks, as well as from oxidative damage to DNA (which is also relevant to key carcinogen characteristic #5)²⁴. It is very important to perform genotoxicity studies to avoid human exposure to the potential DNA

damage that can be caused by genotoxic carcinogens. Mutagenesis (the formation of mutations in DNA molecules) is driven by DNA damage resulting from genotoxicity.

Genotoxicity in animal tissues and cells

There is overwhelming evidence that NDMA is genotoxic, mutagenic and clastogenic (IARC, 1978). NDMA is a well-studied prototype genotoxic carcinogen which binds directly to DNA generating DNA adducts, DNA damage, and mutations¹³⁴. NDMA has been classified as a genotoxic carcinogen by the European Union (ISZW99). NDMA exhibits genotoxic activity in standard assays such as the Ames assay and the Comet assay. Many studies show that NDMA is genotoxic both *in vivo* and *in vitro*. Increased frequencies of gene mutations, chromosomal damage, sister chromatid exchange and unscheduled DNA synthesis have been observed in a wide variety of cell types and assays. Clear evidence of genetic effects has also been observed in *in vivo* studies. Clastogenic effects (e.g., micronuclei, sister chromatid exchange, chromosomal aberrations) in hepatocytes¹³⁵⁻¹³⁷, bone marrow cells^{138,139}, spleen cells¹⁴⁰, and peripheral blood lymphocytes¹³⁶, as well as in esophageal¹⁴¹ and kidney cells¹⁴², have been observed in rodents (rats, mice or hamsters) administered NDMA either orally or by intraperitoneal injection.

Increased frequencies of micronucleated cells were observed at NDMA doses as low as 5 mg/kg body weight in rats¹⁴¹. Effects in germ cells (e.g., micronucleated spermatids) were observed in mice given 6 or 9 mg NDMA/kg body weight via intraperitoneal injection¹⁴³. The inhalation exposure of female mice to NDMA at 1030 mg/m³ increased the frequency of micronucleated bone marrow cells¹⁴⁴. Evidence of genotoxicity (e.g., chromosomal aberrations, micronuclei, gene mutation, DNA strand breaks) has also been observed in the offspring of hamsters¹⁴⁵ and mice¹⁴⁶ administered NDMA during gestation. Numerous publications have demonstrated that in rodents (rats, mice or hamsters) administered NDMA either orally or by

intraperitoneal injection, evidence of DNA damage has been observed in many tissues throughout the body including liver, kidneys and lungs¹⁴⁷⁻¹⁶². DNA damage in the thymus¹⁴⁸, sperm¹⁵⁴, and nasal and tracheal cells¹⁵⁶ has also been observed. NDMA was also mutagenic at the *lacI* locus (in the liver) in *in vivo* assays involving transgenic mice¹⁶³⁻¹⁶⁵. In addition, increased unscheduled hepatic DNA synthesis has been observed in rats at NDMA doses as low as 0.1 mg/kg bw¹⁵⁰. In mice, NDMA caused an increase in mutations (e.g., 10-20 fold) that increased with time and the number of treatments^{163,164}.

The NDMA-induced genotoxic effects and DNA damage in cancer cells generate reactive oxygen species which can lead to further DNA strand breaks and oxidative DNA damage (which synergizes with key characteristic #5) to initiate and promote cancer. Genotoxicity induced by NDMA is further demonstrated in extrahepatic (outside the liver) tissues of rats by the persistence of DNA damage in the lung, liver, kidney and nasal cavity^{158,159,166}. Remarkably, two notable studies observed genotoxicity in the offspring of animals exposed to NDMA¹⁶⁷. When nursing Sprague-Dawley rats were treated with radiolabeled NDMA and other nitrosamines, the liver and kidney DNA from their 14-day-old offspring that had been nursed over a 24-hour period became labeled with the NDMA and other nitrosamines. Notably, upon analysis, liver DNA from the offspring whose nursing mothers were treated with the radiolabeled NDMA showed N7-methylguanine and O6-methylguanine DNA adducts¹⁶⁷.

Importantly, NDMA is such a potent genotoxic carcinogen that it does *not* exhibit a no-observed-adverse-effect level (NOAEL) as demonstrated by studies including Peto *et al.*¹⁹. The NOAEL is defined by the level of exposure of an organism, found by experiment or observation, at which there is no increase in the frequency or severity of any adverse effects (e.g., cancer incidence) of the tested protocol. In Peto *et al.* there was no indication of any threshold

for tumor induction. NDMA at 0.1 ppm in drinking water caused about 2.5% of animals to develop liver tumors and therefore a dose of 0.01 ppm would yield a 0.25% incidence.

NDMA causes genotoxicity in large animals

NDMA causes genotoxicity (DNA damage) in a wide spectrum of tissues in the monkey, with at least eight tissues sustaining DNA damage levels with 50% of those of the liver¹²¹. Among these tissues are included common sites of human cancer such as the large and small bowel, the pancreas and the esophagus. Four hours after NDMA exposure, DNA adducts (O6-meG) are expressed in all tissues including gastric mucosa, liver, white blood cells, esophagus, ovary, large intestine, bladder, spleen, uterus, and brain¹²¹. In the diet, DNA adducts from NDMA (e.g., O6-meG) were found in blood cell DNA, at levels ranging 0.02– 0.12 fmol mg DNA. To quote from this publication ***“It is evident, that in contrast to rodents, these important cancer targets share with liver an equal likelihood of sustaining DNA damage after NDMA exposure¹²¹.”*** Thus, NDMA can lead to cancers and genotoxic damage in many organs and target tissues¹²¹.

Human tissue and cells

Two studies by Hakura *et al.* found that fractions from human cells were considerably more active than those from rats in stimulating the mutagenic response to NDMA in the Ames test: the mutation rate was up to 8 times higher with some human S9 fractions^{168,169}. The recent observation that human S9 fractions are much more active than rat S9 fractions in promoting genotoxicity in the Ames test suggests that humans may be especially sensitive to the carcinogenicity of NDMA^{168,169}. Thus, the genotoxicity of NDMA has been extensively proven in animal models as well as animal and human tissue.

Importantly, human tissues (e.g., liver, kidneys, lung, and digestive tract) metabolize NDMA into genotoxic DNA adducts. Seven different human tissues such as trachea, lung,

esophagus, colon, pancreas, bladder, and buccal mucosa can metabolize NDMA into cancer causing metabolites (e.g., reactive electrophiles- a key characteristic of carcinogens). Human tissues such as human lung (e.g., bronchi), colon and esophagus metabolize NDMA into reactive electrophilic metabolites that react with cellular DNA and protein leading to carcinogen DNA adducts (e.g., alkylated DNA in both O-6 and N-7 position of guanine). Both human liver and lung can metabolize NDMA *in vitro*. Montesano and Magee reported that slices of human liver can metabolize radio-labeled (^{14}C) NDMA into (^{14}C)CO₂ and intermediates that alkylate bases in nucleic acids forming primarily 7-methylguanine¹²⁵. Slices of human lung can metabolize (^{14}C) NDMA, as measured by production of (^{14}C)CO₂. (^{14}C)NDMA binds to both DNA and protein of cultured human bronchi. Binding to DNA was NDMA dose-dependent. NDMA results in methylation of DNA at both the O-6 and N-7 positions of guanine in human cells. NDMA induced O-6 methylation of DNA in cultured human bronchia. Thus, human tissues (e.g., lung tissue) can metabolize NDMA into reactive intermediates.

The cellular and molecular changes induced by nitrosamines (e.g., NDMA) in animals are similar to those in human tissues. Repair of DNA lesions such as O6-methylguanine occurs in other tissues besides the liver. Thus, the cancer-causing mechanisms which occur in animals also take place in humans. The metabolism of NDMA and NDEA as measured by alkylation of DNA is similar in the rat and human esophagus as well as extrahepatic tissues.

NDMA is genotoxic in various human tissues (e.g., liver, colon, bronchi, and esophagus) in cultured cells. DNA molecules in liver cells from humans intoxicated with NDMA contained the methylated purines N7-methylguanine and O6-methylguanine, indicating alterations in DNA caused by NDMA. Liver cancer cells are capable of metabolizing NDMA to genotoxic products.

NDMA induces a dose-dependent increase in the frequency of DNA single-strand breaks and alkali-labile sites in primary cultures of rat lung cells and in lung cells from human donors.

Conclusion – Key Characteristic #2

Abundant studies have demonstrated potent genotoxic activity of NDMA in numerous *in vivo* and *in vitro* assays, including with human cells. Thus, studies with human cells, as well as studies of animals and microbes, clearly demonstrate the genotoxicity activity of NDMA.

It is biologically plausible that NDMA causes cancer via key characteristic #2.

Carcinogen Key Characteristic #3: NDMA Alters DNA Repair and Causes Genomic

Instability

General Description

Normal cells try to avoid deleterious mutations by replicating their genomes with high accuracy. Most spontaneous mutations are caused by polymerase error¹⁷⁰. The nature of the mistake, the presence of DNA damage, and the ability to correct errors all have an impact on the outcome of this process. As a consequence, defects in processes that determine DNA replication fidelity can confer strong mutator phenotypes that result in genomic instability. Thus, carcinogens may act not only by producing DNA damage directly but also by altering the processes that control normal DNA replication. Different susceptibilities of organs to carcinogenic stimuli may be determined by the ability to repair certain alterations produced by the carcinogen in DNA¹⁰².

Why is genomic instability important to the mechanism of carcinogenesis?

DNA damage is a source of genomic instability without correct DNA repair. DNA repair is critical for cancer prevention as DNA repair prevents the genetic mutations in normal cells¹⁷¹. While DNA excision repair pathways are predominantly error-free and thus protective, double-strand break repair is largely error-prone and may contribute to genomic instability. Genomic

instability is a well-recognized hallmark of many cancers and is considered to be one of the enabling characteristics of cancer¹⁰¹. Markers of genomic instability include chromosome aberrations, gene mutations, microsatellite instability, and apoptosis²⁴. The ability of cells to repair DNA adducts (by removing *O*-methylguanine through the action of a specific *O*-methylguanine DNA-methyltransferase) prior to cell division likely plays a critical role in determining the susceptibility of tissues to tumor development.

NDMA alters DNA repair and causes genomic instability.

The ability of the tissue to repair DNA adducts plays an important role in the mechanism of NDMA causing cancer. Importantly, NDMA alters DNA replications and promotes subsequent DNA damage, thereby priming cells for carcinogenesis. The rate of elimination of DNA adducts may be an important factor in neoplastic transformation by alkylating carcinogens. Following administration to rats of various doses of NDMA, O6-methylguanine (O6-meG) was lost from the DNA of four tissues (liver, white blood cells, lymph nodes, bone marrow). NDMA alters DNA repair enzymes (e.g., AGT) with DNA leading to genomic instability. Following the administration of NDMA, alkylation of DNA occurs in both the liver and kidney but the adducts are more persistent in the kidney. O6-methylguanine-DNA transferase (MGMT) is an enzyme that repairs O6-methyl guanine and other O6-alkyl guanine DNA adducts. Repair of DNA by MGMT (or lack of repair) has a key role in the development of NDMA-induced cancer. A single dose of NDMA induces the inability of the kidney to rapidly repair DNA methylation leading to kidney tumors.

Human tissue and cells

Consistent with carcinogen key characteristics #1 and #3, DNA adducts from NDMA are repaired very slowly in human blood, hence, these DNA adducts can build up in the blood. Intra- and intercellular variations occur in the repair efficiency of O6-methylguanine in human liver

cancer cells (HepG2 cells) treated *in vitro* with NDMA¹⁷². MGMT, the enzyme responsible for the repair of O6-methylguanine DNA adducts, has been detected in the liver of humans. Thus, NDMA results in DNA alkylation adducts which are not repaired correctly leading to cancer.

Conclusion – Key Characteristic #3

NDMA alters the processes that control normal DNA replication or repair of DNA damage. Thus, NDMA induces genomic instability with an increased risk for DNA mutations and other genetic changes during cell division. As a result, NDMA is a probable human carcinogen via alterations in DNA repair capacity and genomic instability.

It is biologically plausible that NDMA causes cancer via key characteristic #3.

Carcinogen Key Characteristic #4: NDMA Induces Epigenetic Alterations (e.g., DNA Methylation)

General Description

The term “epigenetic” refers to all stable changes in gene expression and chromatin organization that are independent of the DNA sequence itself and that can be mitotically inherited over cell divisions. Epigenetic phenomena include genomic imprinting, changes in chromatin and histone modification patterns. Epigenetic alterations are changes in gene expression including DNA methylation. Carcinogens can induce epigenetic changes which can lead to cellular transformation. Epigenetic changes initiate and mediate cancer progression.

Why are epigenetic alterations (e.g., DNA methylation) important to the mechanism of carcinogenesis?

A wide range of known and suspected carcinogens (including chemical, physical, and biological agents) have been shown to deregulate the epigenome.

NDMA epigenetic alterations in animal tissues and cells

NDMA-induces epigenetic alterations in the animal studies. In rats, independent of dose and route of administration, NDMA induced DNA methylation (e.g., N7Guanine and O6Guanine) in whole tissues (e.g., liver and nasal mucosa) after a single injection of NDMA. Several independent studies from various laboratories have demonstrated that NDMA induces DNA methylation in rats¹⁷³⁻¹⁷⁵. The induction of microsomal NDMA demethylase activity was closely related to the increase of DNA methylation by NDMA¹⁷⁶.

DNA methylation can mediate the pro-tumorigenic and cancer-initiating activity of NDMA. The rat nasal mucosa contains relatively high levels of cytochrome P-450 enzymes and these enzymes can catalyze the alpha-hydroxylation of NDMA.

Conclusion – Key Characteristic #4

NDMA may disrupt epigenetic mechanisms as a human carcinogen via DNA methylation.

It is biologically plausible that NDMA causes cancer via key characteristic #4.

Carcinogen Key Characteristic #5: NDMA Induces Oxidative Stress*General Description*

Reactive oxygen species (ROS) are compounds that cause oxidative damage to cellular biomolecules. Oxidative stress including reactive oxygen and nitrogen species (RONS) critically mediate cancer progression by carcinogens and pathogens^{171,177}. ROS are important mediators of oxidative stress. An imbalance between formation of reactive oxygen and/or nitrogen species and their detoxification is commonly referred to as oxidative stress. ROS, which can arise from inflammation, may contribute to genomic instability and, along with other free radical species, play key roles in many of the processes identified as being necessary for the conversion of normal cells to cancer cells^{13,25}. Oxidative stress can lead to oxidative damage to DNA¹⁷⁸. Oxidative stress

is directly related to many other key characteristics of carcinogens, notably #2 and #3 via DNA damage leading to genotoxicity and alteration of DNA repair, as well as others including chronic inflammation (#6) and altered cell proliferation (#10)²⁵. Oxidative stress is also a common occurrence in cancer and can be a critical component of the tumor microenvironment. Oxidative damage is considered a major factor in the generation of mutations in DNA, and greater than one hundred different types of oxidative DNA damage have been identified to date¹⁷⁸. Oxidative damage to DNA may initiate or promote carcinogenesis¹⁷⁸.

Why is oxidative stress important to the mechanism of carcinogenesis?

Experimental, clinical and epidemiological studies have provided compelling evidence that oxidative stress is critical in the initiation and progression of cancer. Oxidative stress causes cellular and molecular events that can cause and promote cancer by causing an imbalance between production and accumulation of ROS in cells and tissues and the ability of a biological system to detoxify these reactive products. ROS can trigger genomic instability. Oxidative damage to DNA can lead to point mutations, deletions, insertions, or chromosomal translocations, which can cause activation of oncogenes and inactivation of tumor suppressor genes, potentially leading to initiation of carcinogenesis. Oxidative stress, reactive chemical species (e.g., ROS), and proliferation stimulate DNA damage and inhibit DNA repair to stimulate tumor progression.

NDMA induced oxidative stress in animal tissues and cells

NDMA induces oxidative stress including oxidative damage to both DNA and lipids^{179,180}. NDMA stimulates ROS and induces a dramatic change in the body weight of animals leading to toxicity. NDMA can cause cancer via an aggressive positive feedback loop between oxidative stress, inflammation, DNA damage, and carcinogenesis. NDMA stimulates inflammation-mediated DNA damage.

NDMA triggers metabolites which can produce mutations in DNA via oxidative stress. This results in high amounts of oxidative stress and production of ROS that further contributes to organ damage and cell death. NDMA stimulates oxidative stress and lipid peroxidation that enhances necrosis (cell death), which initiates mitosis and hepatic regeneration. Thus, NDMA injures cells in various organs and throughout the human body in multiple ways to trigger inflammation via oxidative stress and ROS. The formation of 8-OHdG can be one of the causes of point mutations that contribute to the activation of oncogenes or the inactivation of suppressor genes leading to cancer.

The toxic effect of NDMA greatly influences the biological activity and lifespan of immune cells, including neutrophils, by inducing a respiratory burst and subsequent release of ROS which stimulates oxidative stress. Oxidative stress can stimulate proliferation of tumor cells acting in synergy with genotoxic mechanism including mutations to cause cancer. Oxidative and endoplasmic reticulum (ER) stress stimulates apoptotic cell death and survival factors contributing to the persistent cycle of cell death and tissue regeneration (“Phoenix Rising” pathway) which can lead to tumor repopulation and persistent tumor growth. Chronic inflammation and pro-inflammatory cytokines generated by immune cells in the human body cause hyperplasia, oxidative stress, and act synergistically with DNA damage to drive carcinogenesis. Carcinogens induce reactive oxygen and nitrogen species resulting in oxidative stress and inflammation.

Conclusion – Key Characteristic #5

Thus, NDMA can be a human carcinogen by stimulating oxidative stress via oxygen radical-induced cellular injury.

It is biologically plausible that NDMA is a human carcinogen via oxidative stress - key characteristic #5.

Carcinogen Key Characteristic #6: NDMA Stimulates Chronic Inflammation

General Description

The relationship between inflammation and cancer dates back to 1863 when Rudolf Virchow suggested that chronic inflammation from tissue injury stimulates the proliferation of cells leading to cancer¹⁸¹. Many experimental studies including those from my laboratory have indeed confirmed that inflammation can stimulate or induce tumor initiation, growth, and metastasis^{6-10,13,33,182-186}. Inflammation in the tumor microenvironment is now known as a hallmark of cancer¹⁰¹ and is recognized as a key characteristic of carcinogens^{24,25}.

Why is chronic inflammation important to the mechanism of carcinogenesis?

Inflammation triggers escape from tumor latency and tumor dormancy¹⁸⁷. Inflammation is a critical driver of cancer and metastasis which can occur throughout the entire body. Chronic inflammation including signaling from pro-inflammatory cytokines triggers oxidative stress and genotoxicity leading to DNA damage and cancer cell proliferation. Thus, inflammation can synergize with DNA damage to promote cancer growth. Chronic inflammation also acts as a tumor promoter in various malignancies such liver, prostate, pancreatic, colorectal (CRC), gastric, gallbladder, and esophageal cancers^{185,188,189}. My laboratory has also recently demonstrated that chronic inflammation acts as a tumor promoter^{6,7,10,11}.

NDMA induces chronic inflammation in animal tissues and cells

Inflammation, an initial stage of cancer (e.g., cholangiocarcinoma), can be induced in hamsters exposed to NDMA and a human liver fluke infection¹⁹⁰. NDMA-induced cancer tissues showed significantly higher numbers of inflammatory cells, especially eosinophils, bile duct proliferation and IL-17+ inflammatory cell infiltration compared to normal livers¹⁹⁰. NDMA-induced cholangiocarcinoma in hamsters resulted from increased pro-inflammatory molecules

such as high mobility group B1 (HMGB1), interleukin-8, and 8-OxodG (oxidative DNA damage marker) in the cancer tissues¹⁹¹. HMGB1 is a potent pro-inflammatory factor that can initiate and stimulate inflammation¹⁹².

NDMA can also act as a tumor-promoter in bile duct cancers that are initiated with either infection by *Helicobacter pylori* or *Opisthorchis viverrini* (liver fluke)¹⁹¹. A study by Thamavit showed that 12.5% of hamsters receiving 12.5 ppm of NDMA in drinking water alone exhibited tumors, and 50% of hamsters receiving NDMA plus *O. viverrine* infection developed tumors by 40 weeks¹⁹³. In multiple studies in Syrian hamsters, NDMA stimulates inflammation in the bile ducts^{194,195}. Histopathologic analysis of bile duct tissues from hamsters treated with NDMA showed a high number of inflammatory cells^{194,195}. NDMA induced the infiltration of inflammatory cells around the bile ducts and liver at days 30 and 60¹⁹⁵. Similarly, NDMA administration in rats induces chronic inflammation and liver tumors⁷⁴. In rats, NDMA-induced inflammation from an increase in inflammatory cells called neutrophils induces liver fibrosis¹⁹⁶, which can also act as a tumor promoter. Pro-inflammatory molecules such as connective tissue growth factor (CTGF), α -SMA, type I collagen, MMP-2, and MMP-9 were markedly increased in NDMA-treated mice¹⁹⁷.

NDMA can also induce liver fibrosis in mice that is similar to fibrosis observed in humans through the generation of oxyradicals, which can stimulate tumor growth. NDMA-induced fibrosis leads to oxidative stress and generation of reactive oxygen species, which can promote tumor growth. These processes lead to cellular injury and inflammation that trigger activation and transformation of hepatic stellate cells into myofibroblast-like cells, which initiates excessive synthesis of connective tissue proteins, especially collagens. Uncontrolled and extensive fibrosis results in distortion of lobular architecture of the liver leading to nodular formation and cirrhosis.

The perpetual injury and regeneration process can also result in genomic aberrations and mutations that lead to the development of various cancers including bladder, liver and prostate. NDMA triggers the immune system and activates lymphocytes which in turn produce various pro-inflammatory cytokines such as IL-1 β , IL-6, IL-22, interferon- γ (IFN- γ), and TNF- α . The pro-inflammatory cytokines trigger hepatocytes to activate downstream pro-tumorigenic molecular signaling pathways such as NF-kB and TGF- β .

Human tissue and cells

In human cells called human H69 cholangiocytes, NDMA stimulates the pro-inflammatory enzyme cyclooxygenase 2 (COX-2), which can generate pro-inflammatory lipid molecules called eicosanoids¹⁹⁸. Cholangiocytes are epithelial cells that line the bile ducts, which are small tubes that carry bile from the liver. COX-2 over-expression has been observed in various inflammatory diseases and can be strong promoter of cancer via inflammation¹⁹⁸. In human white blood cells (called peripheral blood mononuclear cells), NDMA stimulates several pro-inflammatory and pro-tumorigenic mediators including TNF- α , IL-1, GM-CSF, and VEGF. NDMA activates mitogen-activated protein kinases (MAPK) in human white blood cells, which promotes cell proliferation and cancer progression.

Conclusion – Key Characteristic #6

NDMA functions as a tumor promoter by stimulating chronic inflammation. Strong interdependent links exist between inflammation, genotoxicity and the induction of oxidative stress and genomic instability. Because NDMA stimulates all of these key characteristics, these mechanisms can act synergistically with chronic inflammation to cause and promote cancer. Thus, NDMA is a human carcinogen.

It is biologically plausible that NDMA is a human carcinogen via chronic

inflammation - key characteristic #6.

Carcinogen Key Characteristic #7: NDMA is Immunosuppressive (e.g., inhibits B and T lymphocytes)

General Description

Immunosuppression is a reduction in the capacity of the immune system to respond effectively to foreign molecules including those on tumor cells. Immunosuppression differs from other mechanisms of carcinogenesis in that immunosuppressive agents may not directly transform normal cells into potential tumor cells²⁴. However, they can promote tumor growth synergistically with other processes that directly transform cells. A classic example of an immunosuppressive drug given before organ transplant (e.g., kidney transplants) is cyclosporine. Epidemiological data from patients with congenital immunodeficiencies, virally induced immunodeficiencies (e.g., HIV-mediated), and from patients treated with immunosuppressive therapies (e.g., organ transplant rejection prevention therapies such as cyclosporine) show that profound immunosuppression is associated with an increased cancer risk²⁴.

Why is immunosuppression important to the mechanism of carcinogenesis?

The immune system in the human body normally functions to protect against invading pathogens and eliminate cancers. Immune cells typically have the ability to detect specific markers on potential tumor cells and clear them before cancer is initiated. Thus, chemicals that cause immunosuppression can increase the risk of cancer and persistent immunosuppression can cause or stimulate cancer.

NDMA induced immunosuppression in animal tissues and cells

In mice, chronic exposure to NDMA induced a marked and persistent immunosuppression of cellular and humoral responses in mice¹⁹⁹. Cellular immunity protects the body from cancer via the activation of immune cells called phagocytes, T cells and the release of cytokines in response to an antigen on a tumor cell. Humoral immunity protects the human body from cancer via substances found in the humors, or body fluids. Chronic exposure to NDMA induces immunosuppression via a reduced cellular and humoral immune response in mice. The persistent NDMA-induced immunosuppression could be reversed after the removal of NDMA from the drinking water.

In mice, NDMA induced suppression of the protective antibody response to a T-cell-dependent antigen, and the lymphoproliferative response to the T-cell and the B-cell mitogens in a dose-dependent manner²⁰⁰. Exposure of mice for 14 days to NDMA by intraperitoneal injection resulted in depressed T-lymphocyte function as measured by T-cell proliferation and suppressed IgM antibody-forming cell response in a dose-dependent manner. NDMA-exposed animals exhibited immunotoxicity via reduced humoral antibody responses, T-cell mitogenesis, and bactericidal activity. NDMA suppressed various measures of humoral immunity. NDMA suppressed the IgM antibody-forming cell response to sheep red blood cells (on day four) in a dose-dependent manner. Reduced host resistance to infectious agents (reduced response to streptococci and influenza challenge) following NDMA administration also indicated suppressed effects on humoral immunity. NDMA blocked T-lymphocyte function as measured by T-cell proliferation. NDMA exhibits immunotoxic effects *in vitro* and in various animal models.

In vivo studies have shown that NDMA modulates the cellular immune response by altering the production and/or maturation/differentiation of bone marrow stem cells into functional macrophages. One of the primary cell targets of NDMA is the B-lymphocyte, which normally generates antibodies that protect the body from cancer. NDMA suppressed IgM antibody-forming

cell response to sheep red blood cells after only 4 days in a dose-dependent manner. Splenocyte proliferation in response to lipopolysaccharide, an inflammatory molecule, was also suppressed by NDMA administration, showing an impaired immune response by NDMA. Thus, NDMA decreases the overall reactivity of both T- and B-lymphocytes, which are important to protect from cancer. NDMA suppressed various measures of humoral immunity. Cellular immune response, monitored by stimulation of cells in mixed lymphocyte reaction (MLR), was markedly suppressed by NDMA, suggesting increased cancer progression due to chronic immunosuppression.

Human tissue and cells

In addition to experimental animal evidence, human studies also support adverse effects of NDMA on certain immune functions. In human blood cells called neutrophils, NDMA activates the pro-cancer PI3K-Akt/PKB and immunosuppressive pathway, which in turn, contributes to the activation of pro-inflammatory transcription factors NF- κ B, c-Jun, and FosB²⁰¹. The expression of Bax and Mcl-1 proteins in autologous peripheral blood mononuclear cells (PBMCs) results in impairment of white blood cell (e.g., leukocyte) function in persons exposed to NDMA. NDMA alters humoral immunity and antibody-mediated host defense mechanisms. White blood cell differentials are indicators of the ability of the body to eliminate infection. A reduction in neutrophils can indicate that the ability of the body to attack and destroy invading bacteria, viruses, and other injurious agents (via phagocytosis) is compromised.

Conclusion – Key Characteristic #7

Thus, NDMA can disrupt the normal host immune response, which would usually protect from cancer progression. The ability of NDMA to be immunosuppressive contributes to its pro-carcinogenic activity. Thus, NDEA notably reduces immunosurveillance²⁰² and causes dysfunction of the immune system, and thereby plays a critical role in carcinogenesis.

It is biologically plausible that NDMA causes cancer via immunosuppression, key characteristic #7.

Carcinogen Key Characteristic #8: Modulates Receptor-Mediated Effects

Numerous carcinogens act as ligands to receptor proteins. Receptor-mediated activation broadly falls into two categories: *a*) intracellular activation, mediated by nuclear receptors that translocate into the nucleus and act on DNA as transcription factors, and *b*) activation of cell surface receptors that induce signal-transduction pathways resulting in biological responses that involve a variety of protein kinases²⁴. Molecular pathways that are regulated through ligand-receptor interaction and are most relevant to carcinogenesis include cell proliferation (e.g., stimulation of the normal proliferative pathways, as is the case for estrogen-dependent tissues and hormone therapy), xenobiotic metabolism, and apoptosis²⁴.

The carcinogen NDMA receptor-mediated activity has not been characterized. Receptor-mediated effects can occur at the cell surface (through ligand-binding) or intracellularly (via the disruption of signaling cascades or actions on nuclear/cytosolic receptors), all of which can modulate transcriptional changes in the nucleus. Thus, since both receptor binding and receptor functional activity of NDMA have not been characterized, it is unknown if NDMA exhibits this characteristic.

Carcinogen Key Characteristic #9: NDMA Causes Immortalization By Transforming

Normal Cells Into Cancer Cells.

General Description

Cancer cells are immortal, and therefore have limitless replicative potential and can divide non-stop. Normal (non-cancer) cells have a limited lifespan and will stop dividing.

Immortalization is associated with stemness, the ability of cells to self-replicate indefinitely.

Why is immortalization important to the mechanism of carcinogenesis?

Cancer cells are immortal and undergo proliferation. If the cancer cells are dividing more rapidly, it means the cancer is faster growing or more aggressive. The rate of cancer cell proliferation can be estimated by doing a Ki-67 test for proliferation. The opposite of immortalization is cellular senescence, a cellular program in which cells stop dividing. Chemical carcinogens including tobacco, PCBs and asbestos promote immortalization and inhibit senescence.

NDMA induced immortalization in animal tissues and cells

NDMA is a carcinogen and is highly toxic to experimental animals by causing severe liver injuries and cancers¹⁹¹. NDMA reacts with rapidly proliferating cells in the terminal end buds of cellular DNA forming DNA adducts, which transform normal terminal end buds to cancer²⁰³. Thus, NDMA induces cell transformation from non-malignant cells to cancer cells¹⁹⁸. In *in vitro* studies, nitrosamines including NDMA can transform normal cells (e.g., fibroblasts) into cancer cells. The mutagenic potential of nitrosamines can be measured using a cell transformation assay *in vitro*, and nitrosamine mixture including NDMA caused a malignant transformation in NIH3T3 fibroblast cells to cancer cells²⁰⁴. NIH3T3 cells were used due to their wide applicability in cell malignant transformation studies²⁰⁴. As stem cells can play a role in cell transformation, NDMA and other nitrosamines can induce damaged cells in the stem cell region of the small bowel^{205,206}.

Human tissue and cells

In human cells called H69 cholangiocytes (bile duct cells), NDMA can induced the transformation of normal H69 cells to cancer-like cells via the overexpression of pro-tumorigenic molecules Cy19, Ki-67 and COX-2.

Conclusion – Key Characteristic #9

NDMA can immortalize the non-tumor (normal) cell into tumor cells.

It is biologically plausible that NDMA causes cancer via immortalization- key characteristic #9.

Carcinogen Key Characteristic #10: NDMA Alters Cell Proliferation, Cell Death or Nutrient Supply (e.g., Angiogenesis)

General Description

Sustained cellular proliferation is a key factor in cancer progression. As summarized in the United States Environmental Protection Agency guidance assessing risk of cancer from early-life exposures (EPA, 2005), more frequent cell division during development can result in enhanced fixation of mutations because of the reduced time available for repair of DNA lesions, while clonal expansion of a mutated cell produces a larger population of mutant cells²⁵. Cell death releases pro-inflammatory signals into the surrounding tissue microenvironment, resulting in recruitment of inflammatory cells of the immune system that can participate in tumor promotion through their influence on cancer cell proliferation and invasiveness. Angiogenesis, in which new blood vessels grow into a tumor, is key to providing nutrients to the cancer. Tumor growth requires angiogenesis to grow¹.

Why is alteration of cell proliferation, cell death or nutrient supply (angiogenesis) important to the mechanism of carcinogenesis?

Cell proliferation: Abnormal proliferation can allow transformed cancer cells to evade usual checkpoints and to continue replication.

Cell death: Apoptotic and necrotic cell death releases pro-inflammatory signals into the surrounding tissue microenvironment, recruiting inflammatory immune cells to the site of trauma, which can enhance cancer-cell proliferation and promote cancer metastasis. In addition, my laboratory recently demonstrated that cell death generated by carcinogens as well as cancer therapy (e.g., chemotherapy and radiation) paradoxically stimulate the growth of surviving tumor cells via inflammation and a storm of pro-inflammatory and pro-angiogenic molecules.

Angiogenesis: Tumor growth is dependent on angiogenesis, the formation of new blood vessels¹. Tumor angiogenesis is stimulated by angiogenic growth factors that stimulate blood vessel growth. For example, pro-angiogenic factors (e.g., CXCL-8/IL-8) and vascular endothelial growth factor (VEGF) stimulate tumor growth and tumor dormancy escape.

NDMA alters cell proliferation, cell death, or nutrient supply (e.g., angiogenesis) in animal tissues and cells

In Syrian hamsters, NDMA stimulates proliferation of cells (e.g., from the bile duct), as confirmed with an increase in a proliferation marker called proliferating cell nuclear antigen (PCNA)¹⁹⁴. RNA analysis using a technique called quantitative real time PCR confirmed NDMA-induced proliferation with cell proliferating genes (e.g., telomerase and c-Ski)¹⁹⁴. Stimulation of cell proliferation is important in the induction of mutations or cancer by NDMA^{207,208}.

A single dose of NDMA causes massive activation of pro-apoptotic (cell death) mechanisms. NDMA induces cell death which can lead to tumor-promoting inflammation. NDMA induces apoptotic cell death via CYP2E1-catalyzed metabolism of NDMA²⁰⁹. The metabolites of NDMA trigger apoptotic cell death in these P450-expressing cells²⁰⁹. The metabolism of NDMA

in the CYP2E1-expressing cell line, GM2E1, causes both DNA methylation and oxidation and support that NDMA-mediated DNA damage plays a key role in NDMA-induced cancer²¹⁰. Hydroxydeoxyguanosine (8-OHdG), a biomarker for oxidative DNA damage, was stimulated by NDMA and lowered by administration of ascorbic acid²¹⁰.

NDMA induces the apoptosis (cell death) of neutrophils and PBMC (human macrophage). NDMA also results in cytotoxic activity and apoptosis in various organs (e.g., large bowel). Oxidative stress (key characteristic #5) plays a key role in the NDMA-induced cell death. Cell death induces a pre-cancer “Phoenix rising pathway” that can stimulate tumor growth. Paradoxically, cell death can stimulate tumor growth via inflammation, pro-inflammatory cytokines and bioactive lipid mediators.

In a bile duct (cholangiocarcinoma) cancer model, NDMA stimulated angiogenesis, the formation of new blood vessels and microvessel density via pro-angiogenic and lymphangiogenic factors (e.g., VEGFC) and their receptors (VEGFR3)²¹¹. This was associated with high VEGFR3 and VEGFC which was significantly associated with angiogenesis and metastasis in human cancer tissues from bile duct cancer patients²¹¹. Thus, NDMA stimulates angiogenesis and cancer spread (e.g., metastasis) in bile duct cancers.

Human tissue and cells

In human cholangiocyte cells of the bile duct, NDMA increases the turnover (proliferation) of cells and the proliferation marker called Ki-67¹⁹⁸. NDMA induces apoptotic cell death of various cells such as human white blood cells (e.g., leukocytes). The pro-apoptotic effects of NDMA were confirmed in human leukocytes indicate an active participation of the cell death molecules TRAIL/DR5 complex and Fas protein²¹². NDMA can also induce the apoptosis of human

neutrophils by regulating the expression of death receptor DR5 as well as through the release of its soluble form (sDR5).

Conclusion – Key Characteristic #10

Thus, NDMA can act as a tumor promoter via stimulating these 3 processes: cell proliferation, cell death, and the vascular supply (angiogenesis) that provides oxygen and other nutrients to growing tumors.

It is biologically plausible that NDMA acts as a tumor promoter via cell proliferation, cell death, and the angiogenesis (vascular supply of nutrients) -key characteristic #10.

As a result, NDMA is a potent and very toxic carcinogen which exhibits 9 out of the 10 key characteristics of carcinogens. Given the indisputable compelling scientific evidence of carcinogenic activity in animals, evidence in human cell and tissue, substantial evidence of genotoxicity, and considerable knowledge on the many biologically plausible mechanisms of carcinogenicity of NDMA, ***NDMA can clearly cause cancer in animals and humans.***

**ANIMAL STUDIES SUPPORT THAT NDMA IS DISTRIBUTED
THROUGHOUT THE MAMMALIAN BODY**

Studies in animals exposed to NDMA intravenously or orally, confirm its distribution in the fluids of all tissues in the body of mammals. NDMA is miscible in water. Non-ionized molecules of low molecular weight that are soluble in water, such as NDMA tend to move freely across biological membranes and be distributed in the body fluids or in highly vascularized organs and tissues and in the extravascular fluid.

In the patas monkey, NDMA causes DNA damage to a wide spectrum of tissues, with ***at least eight tissues*** sustaining DNA damage including the liver, esophagus, stomach, pancreas, small and large bowel, bladder, prostate, lung, kidney. uterus, ovaries and white blood cells. Thus,

NDMA-induced cancer can occur in these tissues and any other tissues as the blood (white blood cells) travels through the body¹²¹. This is particularly significant given the similarities between monkeys and humans. Non-human primate models most closely resemble human anatomy and physiology.

In rats exposed to varying oral NDMA doses for 4 weeks and sacrificed thereafter, Anderson et al. reported the presence of NDMA in the blood, liver, kidney, lung and brain, regardless of the dose given. In pregnant Syrian hamsters, two hours after a subcutaneous dose of 12.5 mg/kg, NDMA was detected in maternal blood, placenta, amniotic fluid and fetus.

These studies demonstrate how widely NDMA is distributed throughout the body and is able to cause cancers at a variety of sites.

SIMILARITIES IN METABOLISM OF NDMA BY HUMAN AND RODENTS

The IARC (1978) evaluated the evidence for NDMA causing cancer and determined “*there is sufficient evidence of a carcinogenic effect of NDMA in many experimental animal species. Similarities in its metabolism by human and rodent tissues have been demonstrated. Although no epidemiological data were available, NDMA should be regarded for practical purposes as if it were carcinogenic to humans.*” Multiple other authoritative agencies have concluded that NDMA is a probable human carcinogen, as there is compelling evidence that human tissue, cells and organ cultures, including liver, buccal mucosa, esophagus, bronchus, liver, colon and urinary bladder are capable of metabolic activation of NDMA into an electrophilic and DNA-binding species leading to cancer. These study results demonstrate the metabolic pathways of NDMA-induced cancer are often qualitatively and quantitatively similar in animals and humans.

Bioavailability of NDMA is Dramatically Higher in Larger Species than in Rodents

There are important differences in the bioavailability of NDMA between rodents and humans. Oral bioavailability is defined as the amount of NDMA that reaches the systemic blood circulation which can transport the NDMA to various tissues throughout the body. The bioavailability of NDMA is higher in larger experimental animals compared to rodents, suggesting that NDMA's potent cancer-causing activity observed in rodents can be even more aggressive and lead to many more tumor types in humans. The level of NDMA in the blood following oral administration is primarily controlled by the amount metabolized in the liver. The fraction of an orally administered dose of NDMA that can be detected in blood (compared to I.V. administration) has been investigated in a number of different species. For example, approximately 8% of an orally administered dose was accounted for in the blood of the rat and hamster, while 49%, and up to 93%, of the administered dose was bioavailable in the monkey (49%), pig (67%) or dog (93%)²¹³⁻²¹⁶. Thus, a high percentage of an orally administered dose of NDMA passes through the liver into the systemic circulation in larger species such as dogs and monkeys compared to smaller species such as rodents. NDMA is extracted efficiently by the liver in rats so the oral bioavailability of NDMA is extracted efficiently by the liver in rats so the oral bioavailability of NDMA in rats is only 8% (e.g., >90% of the NDMA is metabolized in a single pass)²¹⁶.

Swine provide an attractive model for physiologic and pathophysiologic studies because their body size, dietary habits, digestive physiology and other physiologic processes are similar to humans. The pig is an omnivore that will eat almost any feed presented to it. This makes the pig a particularly good model for studying *in vivo* nitrosation²¹⁴. In the swine, 67% of an orally administered dose of NDMA passes through the liver into the systemic circulation. Since the systemic clearance of NDMA in swine is greater than hepatic blood flow, organs other than the liver are involved in the clearance of NDMA. This data suggests that a substantial fraction of the

systemic clearance in a swine is extrahepatic and that the pharmacokinetics of NDMA in higher species are likely quite different from that observed in rodents²¹⁵.

A similar situation (e.g., high bioavailability and clearance exceeding hepatic blood flow) was observed in dogs. The lungs also play an important role in the clearance of NDMA. The bioavailability of the oral dose (i.e., the fraction of the dose that reached the systemic circulation) averaged 93% in beagles²¹⁵. To illustrate this point, chronic administration of low doses of NDMA in the rat results in liver tumors whereas single high doses cause other tumor types (e.g., kidney). This occurs because in rodents when small doses of NDMA are given, the capacity of the liver to metabolize the carcinogen is likely sufficient; hence, the nitrosamine is effectively cleared by the liver in a 'first-pass' effect, leaving little to interact with other organs in the rodent. However, if the rodent is given a high dose, first pass metabolism is saturated and a larger fraction of the dose passes the liver and reaches the systemic circulation.

This has two important consequences: 1) In the rodents, levels of NDMA found in peripheral blood may be significantly lower than those expected on the basis of total NDMA exposure because of the rapid metabolism and effective clearance of the carcinogen by the liver; and 2) in larger mammals like humans, low metabolic activation in the liver can result in *more of the carcinogen being metabolized in other tissues, thereby increasing the risk of cancer developing in those other tissues*. Since a large fraction of NDMA passes through the liver in large mammals such as swine, dogs and monkeys, NDMA can lead to extrahepatic cancers in these large mammals. This is also expected to occur in humans.

Since a larger portion of an oral dose of NDMA in swine, dogs and monkeys reaches the systemic circulation, NDMA can be metabolized in organs other than the liver even if the exposure is a low dose. In higher species such as humans the bioavailability of NDMA is high, and organs

in addition to the liver will be exposed to NDMA. There is also ample evidence that human tissues in addition to the liver can metabolize NDMA and repair of DNA lesions such as O6-methylguanine. This is further evidence of human's ability to metabolize NDMA throughout the body. Therefore, animal studies reporting cancers caused by NDMA exposure in the liver and other organs are relevant to humans.

NDMA bioavailability increases with the size of mammals, suggesting that it would be higher in humans than in rodents²¹³⁻²¹⁵. Regarding the pharmacokinetic profile of NDMA in humans, it is unethical to perform NDMA studies in humans because of its severe toxicity and carcinogenic activity. Thus, drawing comparisons with the animal studies is critical. A comparison of NDMA metabolism across 7 different species indicates higher bioavailability of NDMA in species larger than rodents meaning that extrahepatic metabolism of NDMA likely plays an important role in the clearance of NDMA in humans. Thus, humans that have consumed contaminated valsartan are at higher risk of various tumor types including colorectal/intestinal, esophageal/pharyngeal, gastric, kidney, liver, lung, pancreatic, prostate, bladder, and blood (e.g., lymphoma, leukemia, and multiple myeloma) compared to those observed in rodents (e.g., liver, respiratory tract, kidney, and blood vessels).

DOSE-RESPONSE

The concept of dose-response (also known as biological gradient) references the principle that the rate of disease (in this case, cancer) increases with increasing dose of exposure. In the animal studies, NDMA exhibits a classic dose-response.

A. Dose-Response in Animal Studies

NDMA is characterized by a classic dose-response in stimulating cancer growth in many animal studies. One of the first detailed studies was from Peto *et al.* (1991) demonstrating a classic dose-response between exposure to NDMA and NDEA and the induction of liver tumors in the rat^{19,74}. This study included a total of 4,080 rats. Each of the nitrosamines, NDMA and NDEA, were given at 16 different doses in drinking water for lifetime. The main target organ in the rat for tumor formation by NDMA was the liver, although NDMA also caused lung tumors. A linear relationship was observed at low doses (below 1 ppm) suggesting that a dose of 1 ppm NDMA in drinking water would cause about 25% of the animals to develop a liver neoplasm. This Peto *et al.* study impressively involved multiple doses, several animals per dose, and multiple tumor-types to reflect the rates of cancers observed and expected in the NDMA-exposed groups. The follow-up of rats was performed until natural death occurred, which was in some cases more than 3.5 years (in experimental cancer studies, rats are generally sacrificed after 2 years of life which results in not being able to observe the natural increased rate of death from cancer in the last months of life). Increasing the dose of NDMA promoted the incidence of tumors of the lungs, skin and lymphatic and hematopoietic tissues^{19,74}.

The study primarily focused on the evaluation of liver tumors as a result of the first pass effect in rodents because approximately 90% of orally administered NDMA is metabolized by the rodent liver which makes the liver a target organ in the rodent model. On the other hand, in larger mammals like the monkey, dog or pig, approximately 50% of the orally administered dose is accounted for in the blood. This difference suggests that metabolic activation of NDMA occurs in extrahepatic tissues in larger species which can lead to cancer in many tissues besides the liver, and at higher rates compared to a rodent.

Other animal studies have confirmed the findings of Peto *et al.*, that NDMA exhibits a

classic dose response. For example, NDMA causes primary tumors of the lung in a dose-dependent manner. Some of the rats developed more than one tumor. The incidence of tumor-bearing rats significantly increased with the dose of NDMA. Tumors occurred mainly in the nasal cavity, with the highest incidences in the groups receiving 1.0 and 0.2 ppm NDMA (19 of 36 and 31 of 36 tumor-bearing animals, respectively); in the lowest exposure group, 13 of 36 animals developed nasal tumors. No tumors were observed in control animals not exposed to NDMA. Thus, this study demonstrated NDMA-induced cancer progression is directly proportional to the dose of this carcinogen.

In another study, a clear tumor dose response was evident in the liver. NDMA induced aggressive hepatocellular carcinomas and hemangiosarcomas in a dose-dependent fashion. A dose-related effect was also demonstrated in the induction of pre-neoplastic lesions and the incidence of papillomas and carcinomas. In the higher NDMA dose groups the tumors appeared much earlier. Thus, the dose of administered NDMA regulates the incidence of tumors (e.g., liver) and the timing of when they develop. There are dozens of animal studies that show both NDMA and NDEA are classic-dose response carcinogens^{19,74,97,202,217-225}.

The human epidemiological studies that examine the association between NDMA and cancer also show evidence of dose-response. For example, the Hidajat study, an occupational study of NDMA exposure in the rubber industry, clearly illustrates NDMA's dose-response²²⁶. The study examined the overall increased risk of some individual cancer types with increasing occupational exposure to NDMA. A statistically significant increased risk of all malignant neoplasms with increased exposure to NDMA was identified for the second, third and fourth exposure quartiles (1.32, 1.83, and 2.08 respectively) with a P-value for trend of less than 0.01. The statistically significant increased risk of cancer with increased exposure demonstrates a classic-dose response

relationship. This dose-response is also observed in NDMA dietary studies such as De Stefani, Pobel, La Vecchia, Zhu, Larsson, Keszei, Loh, Zheng, DeStefani, Goodman, and Knekt²²⁷⁻²³⁶.

Given the depth of animal studies, which is reinforced by the Hidajat study, the evidence is overwhelming that NDMA and NDEA both exhibit a classic dose-response.

1. Proportional responses are observed at low doses of NDMA with no safe level as there is not a No Observed Effect Level (NOEL)

Genotoxic chemicals have been assumed to exhibit linear cancer dose-response. A dose-threshold can be defined as:

Biological definition: The dose below which the organism does not suffer from any (adverse) effects from the compound considered.

Experimental definition in cancer: The dose below which no cancer is observed.

The linear no threshold data states that *all doses no matter how low, increase the risk of cancer*.

If a threshold exists, safe levels can be calculated; if there is a linear dose-response, there are no safe exposure levels³⁰. The EPA has acknowledged that NDMA has a linear dose-response with an oral slope factor for carcinogenic risk at 51 milligrams per kilogram per day (mg/kg-day) (EPA IRIS 2002) with no threshold. Because a genotoxic chemical can permanently induce DNA damage and mutations, there is viewed to be no safe exposure threshold or dose with genotoxic carcinogens^{30,31}. This is based on the assumption that even one molecule of genotoxic chemicals may induce a mutation that may cause cancer^{31,237}. Accordingly, genotoxic carcinogens are strictly regulated and not allowed to be used as food additives, pesticides, or veterinary drugs (FAO/WHO 2009)³¹. Importantly, genotoxic carcinogens are regulated under the policy that they have no thresholds or safe dose^{30,31}. Thus, there is likely no completely safe dose with NDMA and NDEA in terms of causing cancer. This is evidenced by the ability of NDMA and NDEA ability to cause

cancer at low doses in experimental animals^{19,64,108,202,225,238}.

The effects from exposure to NDMA, even at low doses, include organ damage and transformation of normal cells to cancer cells. This suggests that pro-cancer non-genotoxic mechanisms such as inflammation and oxidative stress, in addition to accumulation of the DNA adducts (*O6*-meG or *N7*-meG), caused the dose-response of cancers from NDMA. The cancer-causing activity of NDMA, while linearly-related to the dose at very low doses, increased sharply at doses higher than 1 ppm. A threshold for a response to a carcinogen is defined as the dose below which no response occurs. Genotoxic carcinogens such as NDMA are DNA reactive, act as tumor initiators to cause cancer, and are assumed to exhibit proportional responses at low doses. NDMA is a risk to human health, even at low doses of exposure via non-genotoxic mechanisms such as stimulating tumor inflammation, oxidative stress, immunosuppression, and tumor angiogenesis. NDMA is activated by cytochrome P450 2E1 in humans and all animal species. Many genotoxins including NDMA and NDEA exhibit a linear relationship between dose and mutagenic response.

Cancer is the critical endpoint used to quantify the dose exposure–response for NDMA. As NDMA-induced cancer mechanisms in animals also occur in humans, cancer causation observed in animals also applies to humans. While there is a difference in rodents versus larger mammals' bioavailability of NDMA, there are no qualitative differences in metabolic activation (e.g., DNA adducts, key characteristic #1) of NDMA between humans and laboratory animals, and there is no reason to believe that humans would respond qualitatively different. The genotoxicity of NDMA (including formation of critical DNA adducts), for which the weight of evidence is exceedingly consistent and convincing, undoubtedly plays a critical role in cancer causation.

NDMA causes liver cancer in a linear relationship at very low doses and increases sharply

at doses higher than 1 ppm with no threshold. A dramatic increase in the carcinogenicity of NDMA above 1 ppm was also reported in the early studies of Druckrey. Importantly, the US Environmental Protection Agency (EPA), the Agency for Toxic Substances and Disease Registry and the Department of Human Health Services state that NDMA and NDEA are human carcinogens that cause cancer in low concentrations with no threshold. This demonstrates that any dose of exposure in humans of NDMA in the contaminated valsartan can cause cancer in humans.

2. Scaling based on the ratio between surface area to body weight of rodents vs. humans is inappropriate for NDMA because of the mechanism of cancer causation

Scaling for variations in the ratios of surface area to body weight between rodent species and humans is likely not needed for the measures of exposure–response developed on the basis of experimental data in animals, since it is well-established that the carcinogenicity of NDMA is mediated mainly through the generation of an active metabolite (i.e., the methyldiazonium ion) (WHO 2002 NDMA). Quantification of exposure and dose-response for cancer and NDMA is based on studies in laboratory animals. It is biologically plausible that agents such as NDMA which cause cancer in experimental animals also cause cancer in humans. Translating the ratios of surface area to body weight from rodents to humans is not appropriate for measuring the exposure–response, which was developed on the basis of experimental data in animals, since it is highly probable that the cancer causation of NDMA is mediated primarily through the generation of an active metabolite (e.g., the methyldiazonium ion); WHO 2002.

The World Health Organization (WHO), in developing their drinking water guideline for NDMA, used a methodology similar to that used by Canada. However, the major difference was that they did not use an animal-to-human scaling factor. They chose a risk level of 10^{-5} and calculated a guideline value of 100 ng L^{-1} for drinking water, based on a 60 kg adult consuming 3

L of water per day. **Importantly, the WHO argued that scaling based on the ratio between surface area to body weight for rodents vs. humans is inappropriate for NDMA because of the probable mechanism of carcinogenicity.** Because genotoxic carcinogens are mutagenic (causing alterations to DNA and chromosomes) and interact with DNA to produce irreversible genetic changes in target organ cells, there is no dose threshold for their carcinogenic potential, suggesting low doses of NDMA or NDEA can cause cancer. **Thus, because of the NDMA-induced genotoxicity and generation of cancer-causing metabolites, NDMA is a human carcinogen independent of any dose scaling between rodents and humans.**

B. Dose-Response in Humans

Hidajat *et al.* is an occupational study of a large cohort of 36,441 males (aged 35 years or older) in the United Kingdom rubber industry. The study had a 49-year follow up period. NDMA exposure was measured quantitatively via a job exposure matrix. This study reported increased cancer mortality with increasing cumulative exposure to NDMA for the following cancers: bladder, lung, stomach, leukemia, multiple myeloma, non-Hodgkin's lymphoma, esophageal, prostate, pancreas and liver cancer²²⁶. The study used a survival analysis to examine cancer mortality risk and compared cumulative exposure in Quartile IV to that in Quartile I. The study found statistically significant elevated risks for death from bladder, lung, stomach, leukemia, multiple myeloma, non-Hodgkin's lymphoma, esophageal, prostate, pancreas and liver cancers associated with increasing NDMA exposure. The study analysis reported P-values for trend of less than 0.01 for bladder, leukemia, multiple myeloma and prostate. The P-values for trend was 0.36 for lung, 0.01 for stomach, 0.11 for non-Hodgkin's lymphoma, 0.26 for esophageal, 0.42 for pancreas and 0.03 for liver cancer. Notably, bladder, lung, stomach, multiple myeloma, esophageal, prostate and pancreas cancers were found to have a statistically significant increased

risk in Quartile II. The increased risk for leukemia, non-Hodgkin's lymphoma, and liver cancer were found to be statistically significant in Quartile III.

The strengths of this study included the large cohort, a long 49-year term follow-up which would capture most cancer related deaths, controls for confounding effects of age, and there was an objective measurement of NDMA exposure. The weaknesses include that it underestimated cancer cases because not all cancer results in death, did not control for smoking (however, the study indicates that simulations of smoking with the data do not change the results), studied only men, and measured the concentration of NDMA in the air, which does not accurately reflect the dose of NDMA absorbed by the worker as a percentage of the NDMA inhaled is exhaled.

In Hidajat, the cumulative exposure to NDMA associated with the four quartiles are defined as follows: Quartile I, less than 3.12 year $\mu\text{g}/\text{m}^3$; Quartile II, 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$; Quartile III, 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$; and Quartile IV, greater than 9.67 year $\mu\text{g}/\text{m}^3$. These quartile values may be converted into micrograms of exposure by using the cumulative exposure values that define the quartiles, the number of weeks worked per year, hours worked per week and breathing rate²³⁹. When doing this calculation, assuming 48 weeks of work per year, 40 hours of work per week (8 per day x 5 days per week), and a breathing rate of 10 m³ per 8-hour work day^{240,241}, there is a cumulative NDMA uptake of 7,488 micrograms for the lower bound of Quartile II, 14,304 micrograms for the lower bound of Quartile III and 23,208 micrograms for the lower bound of Quartile IV.

While NDMA and NDEA are similarly potent, independent of whether the mode of exposure is inhalation or ingestion (oral) as explained earlier in this report, there is an adjustment that should be made to compare the cumulative doses of NDMA in the Hidajat study with cumulative oral doses. The chemical in question must be examined to determine the percentage of

the inhaled dose that is absorbed into the body as it is well accepted that with most chemicals some of the inhaled dose is exhaled. The percentage which is absorbed varies depending on the properties of the chemical such as particle size, solubility, and site of metabolism.

Since this phenomenon is chemical specific, I conducted a search for studies that would address this issue specifically for NDMA. My search yielded an animal study that addressed this issue which was not surprising as it would be unethical to run a trial with humans inhaling a presumed carcinogen. The Klein *et al.* study measured exhaled NDMA following inhaled NDMA in rats²⁴². Specifically, systematic measurements were taken of exhaled NDMA following 10-minute exposures to inhaled NDMA at concentrations of 1 to 450 µg/L. The study found that approximately 10% to 30% of the inhaled dose was exhaled²⁴². Inhalation exposures are similar in animals as humans as the absorption is through the lungs (no variability due to the first pass effect). Thus, the exhalation percentage of 20% (median value) and absorption percentage of 80% observed by Klein may be applied to humans²⁴².

When applying the absorption percentage of 80% to the cumulative exposure values in Hidajat, you arrive at the following cumulative exposure values for each quartile:

1. Cumulative NDMA exposure of 5,990 micrograms for the lower bound of Quartile II;
2. Cumulative NDMA exposure of 11,443 micrograms for the lower bound of Quartile III;
3. Cumulative NDMA exposure of 18,566 micrograms for the lower bound of Quartile IV.

Significantly, the average ppm in the ZHP valsartan API made with the Zinc Chloride process has been represented by ZHP to be as follows: 65.1 ppm for product code D5191, 63.4 ppm for product code C5355, and 56.7 ppm for product code C5523 (PRINSTON00075838). However, there were

batches of ZHP API with NDMA levels as high as 188.1 ppm that were sold in the United States (ZHP00079913-45, at 9920-9928). If the 188.1 ppm API were made into 320 mg tablets, those tablets would have approximately 60,000 nanograms of NDMA.

By way of illustration, a patient taking 320 mg per day of ZHP valsartan (average contamination level for product code D5191 of 65.1 ppm) would ingest 20,832 nanograms of NDMA per day. This patient would reach the cumulative NDMA exposure for the bound of Quartile II in 300 days (approximately 10 months), and this doesn't take into account the threshold exposure to NDMA that a valsartan patient has because of diet, which is estimated in the United States to range from 0.03 to 0.06 $\mu\text{g}/\text{day}$, depending on age, or 0.08 $\mu\text{g}/\text{day}$ when beer is included⁶⁸.

There are several dietary studies that quantified the exposure of NDMA and/or NDEA and measured the risk of various cancers. Several studies had statistically significant findings of risk of cancer with increasing intake of NDMA. The individual studies that quantified daily dietary exposure to NDMA and/or NDEA are addressed later in this report under the individual cancer types. When evaluating exposure levels that give rise to an increased risk of cancer, I focused on the studies that report a statistically significant increased risk and reported daily doses in terms of micrograms/nanograms. These studies reported NDMA daily doses of the lower bounds of the highest tertile/quartile/quintile. For example: De Stefani Gastric Cancer study - 270 nanograms of NDMA per day; Pobel Gastric Cancer study - 290 nanograms of NDMA per day (75% percentile distribution); La Vecchia Gastric Cancer study - 190 nanograms of NDMA per day; Larrson Gastric Cancer study - 190 nanograms per day; Knekt Colorectal Cancer study - 53 nanograms of NDMA per day (mean); Loh Cancer study - 126 nanograms of NDMA per day (Quartile 4); De Stefani Lung Cancer study - 270 nanograms of NDMA per day; and Goodman Lung cancer study - 700 nanograms of NDMA per day (Upper bound of Quartile III intake).

Notably, these reported daily intake levels are significantly less than the average level of NDMA contamination that would be in a ZHP 320 mg tablet, based on the values reported by ZHP. ZHP reported an average level of NDMA contamination for Workshop W02 of 65.1 ppm which would equal 20,832 nanograms of NDMA in a 320 mg tablet; average level of NDMA contamination of 63.4 ppm for ZHP Workshop 2 which would equal 20,288 nanograms of NDMA in a 320 mg tablet; and average level of NDMA contamination of 56.7ppm for ZHP Workshop 13 which would equal 18,144 nanograms of NDMA in a 320 mg tablet (PRINSTON00075838). These average NDMA contamination levels are approximately 189 to 217 times more than the FDA's acceptable daily limit. The highest level of NDMA contamination reported by ZHP was 188.1 ppm which would equate to over 60,000 nanograms in a 320 mg tablet (over 627 times the FDA's acceptable daily limit) (SOLCO00028261).

The lifetime cumulative exposures of NDMA based on the reported high exposure category in the dietary studies based upon 60-years of daily intake would be as follows: De Stefani Gastric Cancer study – 5,913 micrograms; La Vecchia Gastric Cancer study – 4,161 micrograms; Knekt Colorectal Cancer study – 1,160 micrograms (mean); Larsson – 4,161 nanograms; Loh Cancer study – 2,759 micrograms; De Stefani Lung Cancer study – 5,913 micrograms; and Goodman Lung cancer study – 15,330 micrograms.

While these are examples of cumulative NDMA exposures that resulted in a statistically significant increased risk of cancer, they should not be considered as bright line thresholds that are necessary to meet in order for there to be a causal relationship between NDMA exposure and cancer response. Some of the studies showed statistically significant increased risks at lower tertiles/quartiles/quintiles. It is well understood that individuals show widely variable susceptibility to carcinogenic factors, and the dose-response curve is in fact a reflection of the

tolerance distribution. Each modulating factor divides the population into subpopulations of different susceptibility so that outliers that could be present in a homogeneous population are flattened out. A linear extrapolation of a human cancer risk to low dose might therefore be appropriate under certain conditions, even if the dose-response curve in animals has a strongly sigmoidal shape²⁴³.

NDMA CAUSES HUMAN CANCER

The peer reviewed NDMA animal cancer studies demonstrate that NDMA causes over 10 types of cancer in experimental animal models. The cancers identified by the animal bioassays include liver, kidney, lung, bladder, esophageal, stomach, blood, intestinal, bile duct, testicular, ovarian, nasal, brain, sarcomas and blood vessels. The peer-reviewed scientific literature demonstrates that NDMA follows the same mechanisms of action and damages human tissue and cells similarly to that of animals.

As a physician and cancer researcher, I searched for and reviewed the epidemiological studies which specifically quantified human exposure to NDMA. As there are no randomized control trials (RCTs), the studies available to review were limited to occupational exposure and dietary studies. I reviewed these studies to ascertain if the cancers that I previously identified in the animal and human cell and tissue studies also occurred in humans who have been incidentally exposed to NDMA. When analyzing human epidemiological studies, it was instructive to examine the Bradford Hill (BH) criteria as part of my evaluation of causal relationship. As set forth above in the dose-response section of this report, one of the important studies I identified is the occupational study of the rubber industry authored by Hidajat *et al.*, which quantified levels of NDMA exposure, and evaluated the risk of cancer death from cumulative exposure to NDMA²²⁶.

The Hidajat study looked at the overall increased risk of death from cancer, and the increased

risk of death from the following individual cancers: bladder, lung, stomach, leukemia, multiple myeloma, non-Hodgkin's lymphoma, esophagus, prostate, larynx, brain, pancreas and liver. A statistically significant increased risk of cancer death with increasing exposure of NDMA of all malignant neoplasms was identified for the second, third, and fourth exposure quartiles (1.32, 1.83, and 2.08 respectively) with a P-value for trend of less than 0.01²²⁶. The increased risks of death associated for each of the cancer types is addressed below.

When using the Bradford Hill (1965) criteria of causation, I weighed the evidence understanding that the BH criteria are not a checklist in which all must be met in order to identify a causal relationship. As Bradford Hill himself explained: "What I do not believe—and this has been suggested—is that we can usefully lay down some hard-and-fast rules of evidence that must be obeyed before we accept cause and effect. None of my nine viewpoints can bring indisputable evidence for or against the cause-and-effect hypothesis and none can be required as a *sine qua non*."

The Bradford Hill criteria are the following: strength of association, consistency across populations, specificity, temporality, dose-response (biologic gradient), plausibility, coherence, experiment, and analogy³⁹. These criteria as applied to whether the NDMA in valsartan-containing drugs can cause human cancer can generally be described as follows:

- 1. Strength of association/Statistical significance.** If the risk of developing cancer is higher in persons with more exposure to NDMA, then that increases the likelihood of causality and that the association is not due to chance alone. To evaluate strength of association, I reviewed epidemiology studies, occupational and dietary, that quantified the amount of NDMA or NDEA exposure. I found these studies of greater value, as they are the best evidence of the relationship between NDMA and/or NDEA exposure and human cancer.

2. **Consistency of the association.** A consistent association would be one that has been repeatedly observed in various populations, places, circumstances, and times. This criterion is meaningful when evaluating animal cancer studies as well as the available human epidemiology.
3. **Specificity of the association:** If exposure to a chemical only causes a specific disease, then its causal link to that disease can be strengthened. However, Bradford Hill recognized that this criterion cannot be overemphasized as “one-to-one relationships are not frequent” (Hill 1965)²⁴⁴. Carcinogens are known to often cause multiple types of cancer. For example, smoking is an accepted cause of multiple cancers.
4. **Temporality:** Did the exposure take place before presentation of the disease?
5. **Biologic gradient:** This refers to whether there is a demonstrated dose-response. Does the risk of cancer increase with increasing amount of exposure? Exposure can be defined by amount or duration, or combination. If the risk of cancer does increase with exposure, then there is an increased likelihood of a causal relationship. However, one needs to consider that with genotoxins, like NDMA, there is no safe dose, as each exposure has the potential to do permanent damage to the DNA which can cause cancer³⁰.
6. **Plausibility:** Is the association between cancer and NDMA biologically plausible? While biological plausibility does not require proof of mechanism, the mechanisms in which NDMA causes cancer was extensively addressed in the discussion of NDMA exhibiting 9 out of the 10 key characteristics of a carcinogen. Mechanistic evidence can add biological plausibility to epidemiological findings which strengthens causal inference. In evaluating experimental animal studies, mechanistic studies can provide valuable data to address the similar response between experimental animals and humans. This helps to identify the

mechanisms contributing to the induction of the observed animal tumors from carcinogens and to determine whether analogous mechanisms may be operative in humans.

7. **Coherence:** The cause-and-effect interpretation of the data should not seriously conflict with the known facts and the biology of cancer.
8. **Experiment:** As explained, randomized controlled trials (RCTs) can provide strong support to observational evidence, but because NDMA is a known carcinogen it would be unethical to conduct a human clinical trial.
9. **Analogy:** Bradford Hill states that in some circumstances it would be fair to judge by analogy. For example, in this case, N-nitroso compounds, which includes NDMA, are known to exhibit extremely high carcinogenic potency. Nitrosamines all have a similar generic chemical structure with the essential feature being the N–N=O structure. The Report on Carcinogens, 14th Edition of the National Toxicology Program and Department of Health and Human Services lists all of the following 15 N-Nitrosamines as reasonably anticipated to be human carcinogens: N-Methyl-N'-nitro-N-nitrosoguanidine, N-Nitrosodi-n-butylamine, N-Nitrosodiethanolamine, N-Nitrosodiethylamine, N-Nitrosodimethylamine, N-Nitrosodi-n-propylamine, N-Nitroso-N-ethylurea, 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone, N-Nitroso-N-methylurea, N-Nitrosomethylvinylamine, N-Nitrosomorpholine, N-Nitrosornicotine, N-Nitrosopiperidine, N-Nitrosopyrrolidine, and N-Nitrososarcosine. An analogy can be drawn regarding the carcinogenic nature of NDMA as it is in the N-Nitrosamines class of chemical compounds which are all considered to be reasonably anticipated human carcinogens. Given that the class of N-nitroso compounds is known to be carcinogenic, this lends support to a finding a causal relationship between NDMA exposure and human

cancer.

After completing this comprehensive review, I identified the following cancers, that in my opinion, NDMA exposure can cause in humans: liver, bladder, blood (multiple myeloma, non-Hodgkin's lymphoma and leukemia), colorectal/intestinal, esophageal, gastric, kidney, lung, pancreatic, and prostate. My analysis of each of these cancers is discussed in detail below.

There were two epidemiology studies that I identified regarding NDMA in valsartan. The first was Pottegard published in 2018 which studied 5150 people that were followed for a median of 4.6 years²⁴⁵. The study reported an adjusted hazard ratio for overall cancer was 1.09 (95% CI: 0.85 to 1.41), with no evidence of a dose-response relationship ($P=0.70$). For single cancer outcomes, a non-statistically significant increase in risk was observed for colorectal cancer (hazard ratio 1.46, 95% confidence interval 0.79 to 2.73). The authors of this study acknowledge that this study had limitations. First, the study was not able to conclusively identify users of contaminated valsartan and the degree of their use. The groups were "probably contaminated" and "possibly contaminated", and were evaluated by daily dose of valsartan, not amount of NDMA, so they failed to take into account the varying amounts of contamination across manufacturers. There was no control for patients switching from contaminated valsartan to lesser or non-contaminated valsartan or vice versa. There was no control that measured the amount of NDMA contamination in which the patient was exposed. There was also inadequate follow-up to capture all cancer cases.

The second study was Gomm which reported there was no association between exposure to NDMA-contaminated valsartan and the overall risk of cancer, and a statistically significant association between exposure to NDMA-contaminated valsartan and hepatic cancer (adjusted HR 1.16; 95% confidence interval [1.03; 1.31]), but no other individual cancers²⁴⁶. This study had similar limitations to Pottegard *et al.* The study had an insufficient follow-up period (median of 3

years) which is not long enough to capture all cancer cases. Patients were included in the cohort if they filled one prescription of valsartan from 2012-2017, and were assessed based upon their valsartan dosage. This is not a sufficient method to measure the amount of NDMA exposure. The relevant inquiry is the amount of NDMA or NDEA the patients were exposed to, not the amount of valsartan.

Given the limitations in both of these studies, I afforded them little weight in my analysis.

Cancer Type #1 - NDMA Causes Liver Cancer

1. Strength of association / Statistical significance.

Since there are no human RCTs with NDMA or NDEA, I looked to occupational and dietary studies that quantified the amount of NDMA and/or NDEA in which the cohort or study subject were exposed. Studies that quantified the amount of NDMA and/or NDEA exposure in my opinion would be more valuable in assessing the causal link between NDMA in valsartan containing drugs and human cancer.

The study of Hidajat *et al.* (2019) examined cancer mortality risk by following a cohort of 36,441 male rubber industry workers from 1967 through 2015. Prior to the conclusion of the study, 94% of subjects died. The study calculated their occupational exposure to rubber dust, rubber fumes, and N-nitrosamines²²⁶. Exposure to nitrosamine and NDMA exposure were measured quantitatively using a job exposure matrix. NDMA exposure was calculated for each of the workers and the cohort was divided into four equal quartiles based upon cumulative exposure.

The strengths of this study include: a quantitative analysis of NDMA exposure that did not rely on questionnaires and recall; large cohort size of 36,441 workers; long follow-up period which would capture most cancers as most workers were followed until death; and controlled for confounding effects of age. Weaknesses of this study include: cancer mortality studies

underestimate the number of cancer cases because not all cancer causes death; did not control for smoking but study indicates that simulations of smoking with the data do not change the results; only males in the study; measure of the exposure to NDMA in air concentration overestimates actual exposure as only a percentage of the inhaled dose is absorbed (does not account for exhalation of the carcinogens). Given the fact that the study design would underestimate the number of cancer cases, the study provides a conservative measure of the risks associated with NDMA exposure.

In Hidajat, it was found that liver cancer was the cause of death for 122 study subjects²²⁶. A significant strength of association was observed as the men in the third and fourth quartiles had increasing risks of dying of liver cancer (SHR-Subdistribution hazard ratio - 1.96, 95% CI:1.16 to 3.29) and SHR 2.86, 95% CI: 1.78 to 4.59, respectively) compared with men in the lowest quartile of NDMA exposure. A dose-response effect is supported as the P-value for trend was 0.03.

Gomm et al. reported a statistically significant association between exposure to NDMA-contaminated valsartan and hepatic cancer (adjusted HR 1.16; 95% confidence interval [1.03; 1.31]) but for the reasons I set forth above, I afforded this study little weight in my analysis.

In summary, my analysis is that Hidajat strongly supports this Bradford Hill (BH) criterion of causation given the statistically significant increase of cancer death in the more exposed groups (second, third and fourth quartiles) as compared to the lesser exposed group (first quartile). I place significant weight on this criterion as statistically significant findings of increasing risk strengthens casual association.

2. Consistency of the association: Given the lack of RCTs and incidental human exposure studies that quantify the exposure to NDMA, I thought it would be instructive to examine the consistency of the association between NDMA exposure and cancer across mammalian species.

NDMA has been shown to be a potent carcinogen by many scientists across multiple animal species. Many animal studies demonstrated that NDMA induces liver cancer in various animal species including mice, rats, hamsters, rabbits, guinea pigs, and fish. Here are some examples:

NDMA induces cancer via exposure from various routes of administration, such as oral, inhalation, subcutaneous, intraperitoneal injection, intramuscular injection, and exposure via tank water in frogs and fish. Here are 32 representative scientific publications: NDMA is a powerful liver carcinogen in animals, as Magee and Barnes first reported in 1956 that oral administration of NDMA induced liver tumors and metastases *between 26 and 42 weeks* in 19 of 20 rats⁸⁹. In Magee and Barnes (1962), NDMA in the diet induced liver tumors *after 26 weeks* in 13 of 18 rats⁹⁰. In studies by Mohr et al (1974) in hamsters, NDMA induced malignant tumors of the liver including haemangioendotheliomata and hepatocellular carcinoma *after 13 to 28 weeks*²⁴⁷. In Anderson *et al.* (1986), NDMA induced liver tumors *by 16 to 28 weeks* in mice²⁴⁸. In Toth *et al.* (1964), a single injection of NDMA induced liver tumors *after 141 days* in mice²⁴⁹. A single subcutaneous injection of NDMA to adult and newborn BALB/c mice induced hepatomas, hemangiomas and hemangiosarcomas mainly in the liver, and liver cell carcinomas²⁴⁹. In 1967, Terracini et al. showed that NDMA administered in the diet of rats (0, 2, 5, 10, 20 or 50 ppm) for up to 120 weeks resulted in a marked increase in liver tumors in a dose-dependent manner⁸⁴. In Otsuka et al. (1971), NDMA in the diet induced liver (e.g., hepatocellular) tumors *after 5 months* in mice⁸¹. NDMA induced benign and malignant hemangiomas of the liver⁸¹. NDMA is a well-established liver carcinogen in rodents based on statistical analysis of results from Peto et al., in a long-term carcinogenicity test at low doses in a study with 4080 rats¹⁹. Similarly, in a study of 5120 rodents, NDMA induced liver tumors in rats and mice including hemangiomas in the liver⁶⁴. Incidences of liver cancer are generally very high (often 50% to 100%) in rats and hamsters because NDMA and

other nitrosamines are effectively cleared by the liver in a “first-pass” effect, leaving very little to interact with other organs. The NDMA-induced liver tumors in Peto et al. are hepatocellular carcinomas and hemangiosarcomas^{19,64,74}.

In a high profile publication in the high impact prestigious journal *Nature*, a single administration of NDMA induced liver (hepatoma) tumors in newborn and 1-week old rats⁷⁵. Exposure of 0.4 mg NDMA/rat 5 times weekly for 24 weeks (total dose 48 mg/rat) induce liver tumors (3 liver-cell carcinomas, 2 sarcomas) in 5 out of 19 Sprague-Dawley rats²⁵⁰. NDMA also induced liver cancer incidence in male rats receiving 5 parts per million (ppm) in their drinking water for 30 weeks²⁵¹.

NDMA induced liver tumors (e.g. hemangiosarcomas and hemangioendotheliomas) in 97% of mice as compared with control values of 4% after oral administration in drinking water⁹⁶. Malignant hemangioendotheliomas of the liver were observed in mice fed a diet containing 50 ppm of NDMA for 5 months²⁵². Oral administration of NDMA induced adenomas of the liver in 16 cases, carcinomas of the liver in 6 cases, hemangioendothelial sarcomas of the liver in 14 cases in mice²⁵³. Continuous NDMA administration resulted in vascular tumors in the livers of C3Hf female mice, whereas C3Hf males developed a high incidence of hepatomas both after continuous treatment and after a single injection of NDMA²⁵⁴.

NDMA in the drinking water of C3HI male mice resulted in a high liver-tumor incidence²⁵⁵. NDMA induced liver hemangiosarcomas in Balb/c mice, a strain which has a low spontaneous incidence of tumors⁹⁸. NDMA also induced liver cancer including hemangiomas, hemangioendotheliomas, and hemangioendothelial sarcomas in 96% of mice after oral administration⁹⁷. Thus, oral administration of NDMA in mice induces hemangiomas (e.g. hemangioendothelioma) of the liver^{82,83,249,252,256}. There was a positive dose-response in a study

where six intraperitoneal injections of NDMA (1-4 mg/kg bw) administered to 7-day old mice increased hepatomas, hepatocarcinomas, lung adenomas, and hemangiomas²⁵⁷. Six or seven injections of 16 mg/g body weight of NDMA, administered over a period of 3-4 weeks, gave rise to liver tumors, both anaplastic and nodular hepatic cell tumors²⁵⁸.

Balb/c mice and Wistar rats were exposed to daily inhalation of NDMA for 17 months (for mice) and 25 months (for rats). Tumors were observed earlier and were larger in the liver in the mice given the concentration of 0.2 mg/m³ compared to control animals⁷⁸. NDMA and NDEA dissolved in tank water at a dose of 5 or 50 ppm, induced liver tumors (hepatocellular cancers and adenomas) in 19 of 43 frogs (44.2%)⁷⁶.

Guinea-pigs given 25 or 50 mg NDMA in the diet for 6-49 weeks developed liver-cell carcinomas⁹². Doses of 7.5 to 1920 mg NDMA/100 g dry diet for 9 months or longer induced liver cancers (adenomas and adenocarcinomas) in rainbow trout²⁵⁹. These cancers were grossly and histologically similar to those reported for rats and other mammals, with direct spread of the neoplasms from the liver to visceral fascia and to pancreatic adipose tissue in a subset of trout²⁵⁹. In fish, NDMA induced liver tumors *after 9 to 20 months*²⁵⁹. Guppies developed liver tumors after *13 months* of exposure to NDMA²⁶⁰.

In larger animals such as rabbits, Le Page et al. (1969) demonstrated that NDMA induced liver tumors with metastases after 23 weeks⁹¹. Rabbits fed low concentrations of NDMA developed liver cirrhosis and liver cell tumors⁹¹. Doses of 25 mg and 50 mg NDMA/kg in the diet of rabbits for 17-60 weeks induced hepatocellular cancer with lung metastases and benign papillary cholangiomas⁹¹.

The Hidajat study together with the consistent association between NDMA exposure and liver cancer across many mammalian species including mice, rats, hamsters, rabbits, guinea pigs,

and fish, studied by several researchers using different study designs over several decades is significant and gives strong support in favor of a causal association.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. Exposure to NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood, and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer, so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA suffered increasing incidents of death from liver cancer. The NDMA occupational exposure quartiles were defined as follows: Quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$; Quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, Quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and Quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of liver cancer increased: Quartile II 1.53 (0.93,2.50) Quartile III 1.96 (1.16,3.29), and Quartile IV 2.86 (1.78,4.59), with a P for trend of 0.03.

The animal cancer studies show a strong dose-response association between NDMA exposure and liver cancer: increasing rate of liver cancer with increasing dose of NDMA. The largest study that illustrated this classic dose response was the Peto, *et al*, study entitled, “*Effects on 4080 Rats of Chronic Ingestion of N-Nitrosodiethylamine or N-Nitrosodimethylamine: A Detailed Dose-Response*

Study” (1991)¹⁹. The purpose of this study was to characterize the dose-response relationship for the effects of NDEA on esophageal cancer, and NDMA and NDEA on liver cancer. NDEA-induced cancers of the nasopharynx and NDMA-induced cancers of the lung were also observed. The four principal dose-response relationships studied were of NDEA on esophageal or liver cells and of NDMA on bile duct or liver cells. At low dose rates, the number of liver neoplasms induced was simply proportional to the dose rate. For example, a dose of 1 ppm of NDEA or NDMA in the drinking water caused about 25% of the test animals to develop a liver neoplasm and a dose of 0.1 ppm caused about 2.5% of the test animals to develop a liver neoplasm. Importantly, there was no indication of any “threshold”, with the higher the dose, the higher the incidence of cancer. These findings of increasing cases of liver cancer with increasing dose show a classic dose-response.

This classic dose response was observed in many other animal bioassays. For example, in 1967, Terracini et al. showed that NDMA administered in the diet of rats (0, 2, 5, 10, 20 or 50 ppm) for up to 120 weeks resulted in a marked increase in liver tumors in a dose-dependent manner. There was a positive dose-response relationship as six intraperitoneal injections of NDMA (1-4 mg/kg bw) to 7-day old mice increased hepatomas, hepatocarcinomas, lung adenomas, and hemangiomas²⁵⁷. Six or seven injections of 16 mg/g body weight of NDMA, administered over a period of 3-4 weeks, gave rise to a short-term lethal effect due to liver necrosis and in the long term to liver tumors²⁵⁸. The tumors induced were of similar type to nitrosamine-induced tumors in rats, one being an anaplastic liver tumor and the other nodular hepatic cell tumors²⁵⁸.

I placed significant weight on this factor as strong evidence of dose-response as in Hidajat and the animal studies strengthens casual association.

6. Bio Plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including human

liver cancer.

In particular, the following studies support the bio plausibility of NDMA causing liver cancer. In a case of suspected NDMA poisoning in a human male, methylation of liver DNA was evident at both the N^7 and O^6 positions of guanine¹²⁶. NDMA also induces DNA damage (e.g., DNA strand breaks and oxidative DNA damage) in human derived liver cancer cells²⁶¹. Even though a much smaller percentage of NDMA is metabolized by the human liver than a rodent's liver, human liver microsomes are as efficient as rat liver microsomes in the metabolism of NDMA²⁶². While NDMA is metabolized is similar mechanisms via metabolic activation such as DNA reactive metabolites (characteristic #1), the first pass metabolism of NDMA is higher in rodents than larger animals resulting in a higher bioavailability in larger animals²¹³⁻²¹⁵.

Importantly, human liver microsomes are capable of metabolizing NDMA via the pathways that have been established with rat liver microsomes^{262,263}. Both human lung and human liver metabolize NDMA via the same metabolic pathway as in animal species. Similar to animals, cytochrome P450 2E1 in human liver microsomes metabolizes NDMA in a similar fashion²⁶⁴. NDMA increased oxidative stress (e.g., reactive oxygen species) and induced genotoxicity in human liver cancer cells²⁶⁵. Animal livers including monkeys, fish, rats, hamsters, and mink can metabolize NDMA into the DNA adducts^{108,121,266-268}. Similar to animals, cytochrome P450 2E1 from human liver microsomes metabolizes NDMA in similar fashion²⁶⁴. NDMA increased the key characteristic oxidative stress (e.g., reactive oxygen species) and caused genotoxicity in human liver cancer cells²⁶⁵. During metabolic conversion of NDMA in human liver preparations, there are no qualitative differences in the metabolism of NDMA between humans and laboratory animals. In addition, there is overwhelming evidence that NDMA is mutagenic, clastogenic, and genotoxic (reviewed in IARC, 1978; ATSDR, 1989). Increased

frequencies of gene mutations, chromosomal damage, sister chromatid exchange, and unscheduled DNA synthesis have been observed in a wide variety of cell types, in assays with human cells. Thus, there are no *qualitative* differences in genotoxicity or metabolism of NDMA between humans and laboratory animals, and there is no reason to believe that humans would respond qualitatively differently to NDMA from animals.

In publication in the high impact journal *Nature*, Montesano and Magee demonstrated that slices of human liver can metabolize NDMA into the DNA adduct 7-methylguanine¹²⁵. They found that human liver slices metabolize NDMA at a rate such that 0.13% of the DNA guanine was methylated at position 7 in 1 hour compared to 0.17% of the DNA guanine in rat liver slices¹²⁵. Humans, like rodents, activate NDMA to a strong methylating agent, resulting in methylation of liver DNA at both the 7- and O6 positions of guanine¹²⁶. Thus, the human liver has the capacity to activate NDMA like that of the rat liver.

NDMA has 9 out of the 10 key characteristics of a carcinogen and there are human liver tissue studies and animal studies showing metabolism of NDMA, so I assigned great weight to this factor in my analysis.

7. Coherence: NDMA-induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). Some ingested NDMA undergoes first pass metabolism in the liver where it is metabolized into an active metabolite by cytochrome P450 enzymes (which are highly expressed

in the human liver)²⁶⁹. Thus, it makes biological sense that the liver would have an increased risk of cancer development after ingestion of NDMA, such as from contaminated valsartan.

The presence of cytochrome P450 enzymes in the human liver showing the metabolism of NDMA by the liver is consistent with the biology of cancer and supports a causal association. As such, I assigned this factor significant weight in my causal analysis.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which include the Hidajat occupational study, NDMA animal cancer studies, human and animal tissue studies, that NDMA in the valsartan-containing drugs increases the risk of and causes liver cancer.

Cancer Type #2 - NDMA Causes Bladder Cancer

1. Strength of association / Statistical significance.

The Hidajat et al. study (2019) previously discussed in detail above found that bladder cancer was the cause of death for 417 study subjects²²⁶. A significant strength of association was observed as the men in the second, third, and fourth quartiles had increasing significant relative risks of dying from bladder cancer (SHR 1.57, 95% CI: 1.19-2.07; SHR 2.45, 95% CI: 1.87-3.21; and SHR 2.82, 95% CI: 2.16-3.67, respectively) compared with men in the first quartile (lowest NDMA exposure). A dose-response effect is strongly supported as the p-value for trend was less than 0.01.

The Jakszyn 2011 dietary study, which quantified the amount of NDMA exposure, examined the incidence of bladder cancer. The study found when comparing the higher exposure group to the lower exposure group, there was a 12% increased risk of bladder cancer, albeit not statistically significant (RR= 1.12, 95% CI: 0.88-1.44). Given the relative risk and range of the confidence interval, the existence of an increased risk is not disproven, and this study is not proof of a lack of a causal relationship.

Hidajat strongly supports this Bradford Hill criterion of causation given the statistically significant increase observed in the most exposed groups (second, third and fourth quartiles) with a P-value for trend of less than 0.01 as compared to the least exposed group (first quartile). I placed significant weight on this factor as a finding of statistically significant increased risk in a well-designed human epidemiological study strengthens causal association, and the one dietary study shows increased risk, though not statistically significant.

2. Consistency of the association: Given the lack of RCTs and human bladder cancer studies that quantify exposure to NDMA, I thought it would be instructive to examine the consistency of the association between NDMA exposure and cancer across mammalian species. NDMA has been

shown to be a powerful carcinogen by many scientists across multiple animal species. There are very limited animal studies in which NDMA is used to cause bladder cancer. However, one study demonstrated that mice developed bladder cancer when exposed to NDMA.

In a Chala et al. bladder cancer model mouse study, NDMA was used as a tumor-promoter with schistosomiasis (*Schistosoma haematobium*) which is used to initiate the bladder cancer²⁷⁰. A urinary bladder cancer model is created by injecting *S. haematobium* eggs into the bladder wall and then adding NDMA²⁷⁰. Inflammation (key characteristic #6), cell proliferation (key characteristic #10) and signs of cancer (e.g., abnormal changes in the bladder tissue such as dysplasia and hyperplasia) were observed in the mice administered NDMA and *S. haematobium* eggs. Vimentin, a pro-cancer marker of epithelial-mesenchymal transition (EMT), was also increased in the mice that received both *S. haematobium* eggs and NDMA; EMT is a pro-cancer process that makes tumors more invasive and able to spread throughout the body more aggressively. The EMT changes after exposure to *S. haematobium* eggs and NDMA coupled with histopathological and *Ki-67* (proliferation) data at the same time point suggest that NDMA can act as a tumor-promoter in this bladder cancer model²⁷⁰. *Vimentin* was observed in 43% of bladder cancers, whereas it was either not expressed or found negative in all normal urothelial cells²⁷¹. The expression of *vimentin* may indicate a high degree malignancy of bladder cancer²⁷¹. Thus, NDMA acts as a tumor-promoter slowly transforming normal bladder cells into bladder cancer cells²⁷⁰.

The association shown in Hidajat and the consistency of the association between NDMA exposure and bladder cancer across many mammalian species, studied by numerous researchers, using different study designs, over several decades is significant and gives moderate support in favor of a causal association.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood, and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from bladder cancer. NDMA occupational exposure was defined for Quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, Quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, Quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and Quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increased exposure, the incidence of bladder cancer increased: Quartile II 1.57 (1.19, 2.07) Quartile III 2.45 (1.87, 3.21), and Quartile IV 2.82 (2.16, 3.67) with a P-value for trend of less than 0.01.

I placed significant weight on this aspect of causality as Hidajat supports a dose-response relationship and the Jakszyn 2011 dietary study showed a non-statistically significant increased risk.

6. Bio plausibility: As set forth in detail in the Key Characteristics of Carcinogens section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including bladder cancer. In particular, the following studies support the bio plausibility of NDMA

causing bladder cancer: The bladder tissue in Patas monkeys is a sensitive target for NDMA induced DNA adduct damage (e.g. O(6)-methylguanine), which can lead to bladder cancer¹²¹. Studies also show that NDMA can be metabolized by human bladder tissues²⁷².

Human bladder cells (e.g., epithelial cells) can activate and metabolize NDMA into DNA binding metabolites as measured by the formation of carbon dioxide (CO₂) and aldehydes. The human bladder cells convert NDMA into carbon dioxide (CO₂) and metabolites such as aldehydes which react with DNA or are incorporated into DNA¹²⁸.

Given that NDMA has 9 out of the 10 key characteristics of a carcinogen, this factor weighs heavily in favor of a causal relationship. There are also animal tissue and human bladder cell studies that support the bio plausibility of NDMA causing bladder cancer. As such, I assigned strong weight to this factor in my analysis.

7. Coherence: NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all blood tissues throughout the body including the bladder. Cytochrome P450 enzymes, which metabolize NDMA into its carcinogenic metabolite, are expressed in the human bladder²⁷³. Thus, it makes biological sense that the bladder would have an increased risk of cancer development

after ingestion of NDMA, such as from contaminated valsartan.

In particular, the following study supports the bio plausibility of NDMA causing bladder cancer: NDMA induced pro-mutagenic DNA adducts (e.g. O6-methylguanine) in the monkey bladder¹²¹. A study of human NDMA metabolism showed that human bladder cells (e.g., epithelial cells) can activate and metabolize NDMA into DNA binding metabolites as measured by the formation of carbon dioxide (CO₂) and aldehydes. In this study, the human bladder cells converted NDMA into carbon dioxide (CO₂) and metabolites such as aldehydes which reacted with DNA or were incorporated into DNA¹²⁸.

The presence of cytochrome P450 enzymes in the human bladder, and studies of animal tissue and human bladder cells showing the metabolization of NDMA is consistent with the biology of cancer and supports a causal association. I assigned this factor significant weight.

8. **Experiment:** As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. **Analogy:** N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the Hidajat occupational study,

NDMA animal cancer studies, animal tissue study, and human tissue and cell studies, NDMA in the valsartan-containing drugs can cause bladder cancer.

Cancer Type #3 - NDMA Causes Blood Cancers (e.g., Multiple Myeloma, Non-Hodgkin's Lymphoma and Leukemia)

1. Strength of association / Statistical significance.

The Hidajat et al. study (2019) previously discussed in detail above found that multiple myeloma, non-Hodgkin lymphoma and leukemia were the cause of death for 462, 141 and 195 study workers respectively²²⁶. A significant strength of association was observed for multiple myeloma in the second, third, and fourth quartiles as the men had increasing significant relative risks of dying from multiple myeloma (SHR 1.59, 95% CI: 1.22-2.08), (SHR 2.78, 95% CI: 2.15-3.60) and (SHR 2.81, 95% CI: 2.17-3.64 respectively) (P-value less than 0.01), compared with men in the first quartile, which had the lowest NDMA exposure. A dose-response effect is strongly supported as the P-value for trend was less than 0.01. A significant strength of association was observed for non-Hodgkin's lymphoma and leukemia in the third and fourth quartiles as the men had increasing significant relative risks of dying from non-Hodgkin's lymphoma (SHR 2.17, 95% CI: 1.35-3.47 and 2.25, 95% CI: 1.41-3.59, respectively) (P-value 0.11) and leukemia (SHR 3.27, 95% CI: 2.20-4.86 and 3.47, 95% CI: 2.35 to 5.13, respectively) (P-value less than 0.01) compared with men in the first quartile, which had the lowest NDMA exposure. This dose-response effect is strongly supported as the P-value for trend was less than 0.01. I did not identify a NDMA dietary study that evaluated blood cancers.

Hidajat strongly supports this Bradford Hill factor of causation given the statistically significant increase seen in the more exposed groups (second, third, and fourth for multiple myeloma, or third and fourth quartiles for non-Hodgkin's lymphoma and leukemia). I placed

significant weight on this factor as a well-designed human epidemiological study supports causal relationship.

2. Consistency of the association: Given the lack of RCTs and incidental human exposure studies that quantify the exposure to NDMA, I thought it would be instructive to examine the consistency of the association between NDMA exposure and cancer across mammalian species. NDMA has been shown to be a powerful carcinogen by many scientists across multiple animal species. There are animal studies showing mice, frogs, and mollusks all developed blood cancers when exposed to NDMA.

Here are some examples: NDMA causes blood cancers including lymphoma and leukemia. In frogs (*rana temporaria*) NDMA (5 mg/l) in tank water induced tumors of the hematopoietic system (“blood tumors”)⁷⁶. Tumors of the hematopoietic system were also observed in mollusks exposed to NDMA in the tank water. NDMA induced leukemia after oral administration in mice⁸¹. Oral administration of NDMA induced leukemia in 3 cases in mice²⁵³. In a high profile publication in the high impact prestigious journal *Nature*, a single administration of NDMA induced lymphoma tumors in rats⁷⁵. NDMA also caused a significant excess of malignant lymphomas in the mice and rats given NDMA. The lymphoma incidence (55%) after NDMA exposure was much higher than untreated controls (4%).

The consistency of the association between NDMA exposure and blood cancer in the Hidajat human occupational exposure study and in the animal cancer studies by several researchers, using different study designs, over several decades is significant and gives support in favor of a causal association which I afforded significant weight.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes

human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood, and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from blood cancer²²⁶. NDMA occupational exposure was defined for Quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, Quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, Quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and Quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of blood cancers increased: Multiple myeloma- Quartile II 1.59 (1.22, 2.08) Quartile III 2.78 (2.15, 3.60), and Quartile IV 2.81 (2.17, 3.64) with a P-value for trend of less than .01; Non-Hodgkin's lymphoma- Quartile III 2.17 (1.35, 3.47), and Quartile IV 2.25 (1.41, 3.59) with a P-value for trend of 0.11; Leukemia- Quartile III 3.27 (2.20, 4.86), and Quartile IV 3.47 (2.35, 5.13) with a P-value for trend of less than 0.01. I placed significant weight on this aspect of causality as Hidajat supports a dose-response relationship²²⁶.

6. Bio plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including blood cancers. In particular, the following study supports the bio plausibility of NDMA causing blood cancer: NDMA induced pro-mutagenic DNA adducts (e.g. O6-methylguanine) in the monkey

white blood cells¹²¹.

There are also human tissue studies which support the bio plausibility of NDMA causing blood cancer. NDMA activates the pro-cancer pathway PI3K-Akt in human leukocytes such as human neutrophils that also stimulate the pro-tumorigenic factors NF-kB and c-Jun, which are associated with more aggressive tumor growth and poor survival²⁰¹. NDMA induces cell death (e.g., apoptosis) in human leukocytes (e.g., peripheral blood monocytes and polymorphonuclear neutrophils)²⁷⁴. Peripheral blood monocytes from humans are sensitive to NDMA via cytotoxic effects²⁷⁵. In addition, human blood cells (e.g. lymphoblastoid cells) are capable of metabolically activating NDMA via NDMA-induced mutations²⁷⁶.

Given that NDMA has 9 out of the 10 key characteristics of a carcinogen and the human tissue and animal tissue studies supporting bio plausibility, this factor weighs heavily in favor of causal risk and I assigned strong weight to this factor in my analysis.

7. Coherence: NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all blood tissues throughout the body. In the blood, NDMA can be metabolized into an active metabolite by cytochrome P450 enzymes expressed by leucocytes (white blood cells)^{277,278}. Thus,

it makes biological sense that there would be an increased risk for blood cancer development after ingestion of NDMA, such as from contaminated valsartan.

O6-methylguanine is detected in human cord blood in mothers highly exposed to NDMA, which implicates NDMA exposure of the fetus¹²⁴. NDMA and DNA adduct formation was detected in the perfused human placenta¹²⁴. This is consistent with epidemiological studies that NDMA may induce brain tumors in children if the mother has consumed large amounts of cured meats during pregnancy²⁷⁹⁻²⁸¹. The Anderson et al. study supports the bio plausibility of NDMA causing blood cancers, NDMA induced pro-mutagenic DNA adducts (e.g. O6-methylguanine) in the monkey blood (e.g. white blood cells)¹²¹.

The presence of cytochrome P450 enzymes in human blood and studies of animal white blood cells showing the metabolization of NDMA is consistent with the biology of cancer, and supports a causal association. As such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific

certainty, based on a totality of the evidence, which includes the Hidajat study, NDMA animal cancer studies, animal cell studies, and human tissue cell studies, that NDMA in the valsartan-containing drugs increases the risk of and causes blood cancers, and in particular, multiple myeloma, non-Hodgkin's lymphoma and leukemia.

Cancer Type #4 - NDMA Causes Gastric Cancer

1. Strength of association / Statistical significance.

The Hidajat et al. study (2019) previously discussed in detail above found that gastric cancer was the cause of death for 768 study subjects²²⁶. A significant strength of association was observed as the men in the second, third, and fourth quartiles had increasing statistically significant risks of dying from stomach cancer (SHR 1.32, 95% CI:1.10-1.57, SHR 1.62, 95% CI:1.32-1.98, and SHR 1.72, 95% CI: 1.41-2.10 respectively) compared with men in the first quartile (lowest NDMA exposure). A dose-response effect is strongly supported as the P-value for trend of 0.01²²⁶.

There have been several dietary studies that quantified the amounts of NDMA exposure and examined the incidence of gastric cancer. Song conducted a meta-analysis study in 2015 which included 11 studies concerning NDMA and gastric cancer and yielded a statistically significant relative risk estimate of 1.34 (95% CI:1.02, 1.76). Of the studies included in the Song meta-analysis, individually the DeStefani, Pobel, LaVecchia, and Larsson studies had statistically significant findings while the Palli (non-significant increase), Loh (non-significant increase), Knekt, Keszei (increase in men), and Jakszyn studies did not. The Loh dietary study found a 13% increase in gastric cancer that was not statistically significant findings (RR=1.13, 95% CI:0.81, 1.57).

I placed strong weight on this factor as a well-designed human occupational exposure study, Hidajat, and large dietary meta-analysis study both support a statistically significant causal relationship.

2. Consistency of the association: There is both a NDMA occupational study and NDMA meta-analysis dietary study that report a statistically significant causal relationship between increasing NDMA exposure and gastric cancer. There is both a NDMA occupational study and I also examined the consistency of the association between NDMA exposure and cancer across mammalian species and discovered a few animal studies that reported NDMA-induced stomach cancer. Here are some examples: Oral administration of NDMA induced forestomach papillomas in seven cases²⁵³. Administration of NDMA in the drinking water induced invasive adenocarcinoma of the stomach in Syrian hamsters⁸⁵.

The consistency of the association between NDMA exposure and gastric cancer recognized across the occupational study, dietary studies and animal studies is significant and gives strong support in favor of a causal association.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA

must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from gastric cancer²²⁶. NDMA occupational exposure was defined for quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of gastric cancer increased: Quartile II 1.32 (1.10, 1.57), Quartile III 1.62 (1.32, 1.98), and Quartile IV 1.72 (1.41, 2.10) with a P-value for trend of 0.01.

I placed significant weight on this aspect of causality as both Hidajat and the Song dietary study meta-analysis support a dose-response relationship.

6. Bio plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including gastric cancer. In particular, the following studies support the bio plausibility of NDMA causing gastric cancer: The digestive tissues (e.g. esophagus, stomach, and large bowel) in Patas monkeys are sensitive targets for NDMA-induced DNA adduct damage (e.g. O(6)-methylguanine),¹²¹ which can lead to digestive cancers. For example, the amount of adducts in the stomach mucosa were consistently higher than the liver¹²¹.

Given that NDMA has 9 out of the 10 key characteristics of a carcinogen and the animal tissue study evidenced the metabolization of NDMA, this factor weighs heavily in favor of a causal relationship between NDMA exposure and gastric cancer, and I assigned strong weight to this factor in my analysis.

7. Coherence: NDMA-induced cancer is consistent with the generally known facts and the

biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). Cytochrome P450 enzymes are expressed in the human stomach²⁸² which metabolize NDMA into a carcinogenic metabolite. Thus, it makes biological sense that the stomach would have an increased risk of cancer development after ingestion of NDMA, such as from contaminated valsartan.

The presence of cytochrome P450 enzymes in the human stomach and animal studies showing the metabolism of NDMA by the stomach cells is consistent with the biology of cancer and supports a causal association. As such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells, all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific

certainty, based on a totality of the evidence, which includes the NDMA animal cancer studies, animal tissue and cell studies, and Hidajat and dietary studies including the Song meta-analysis, that NDMA in the valsartan-containing drugs increases the risk of and causes gastric cancer.

Cancer Type #5- NDMA Causes Intestinal Cancer (Small Intestine, Large Intestine, Colorectal and Rectal)

1. Strength of association / Statistical significance.

The Hidajat et al. (2019) did not evaluate the incidence of intestinal cancer (small intestine, large intestine, colorectal or rectal). However, there are several dietary studies that evaluated colorectal and rectal cancer and found a statistically significant correlation. Zhu found a 42% increase in colorectal cancer (OR 1.42, CI:95% 1.03-1.96)²³⁰. The Knekt et al. study also found a statistically significant increased risk of colorectal cancer of 2.12 (95% CI: 1.04, 4.33)²³⁶. The Loh et al. study found a statistically increased risk of rectal cancer (HR: 1.46; 95% CI: 1.16, 1.84) and reported a non-statistically significant colon cancer hazard ratio of 0.99 (95% CI: 0.83, 1.18)²³³.

In Pottegard, a non-statistically significant increase in risk was observed for colorectal cancer (HR 1.46, 95% CI: 0.79 to 2.73)²⁴⁵. For the reasons set forth previously, the Pottegard study did not carry significant weight in my analysis.

The dietary studies, Zhu (colorectal), Loh (rectal cancer only) and Knekt (colorectal), that quantified NDMA intake support a statistically increased risk of colorectal or rectal cancer with increasing exposure to NDMA which satisfy this criterion. I placed strong weight on this factor.

2. Consistency of the association: When looking at this Bradford Hill factor, I thought it would be instructive to examine the consistency of the association between NDMA exposure and cancer across mammalian species. NDMA has been shown to be a powerful carcinogen by many scientists across multiple animal species. The consistency of the association between NDMA

exposure and intestinal cancer in the dietary studies, using different study designs, over several decades is significant and gives support in favor of a causal association which I afforded significant weight.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In the dietary studies of Knekt, Zhu and Loh (for rectal cancer only), increasing exposure to NDMA resulted in increasing cases of colorectal or rectal cancer. I placed strong weight on this aspect of causality as multiple human dietary studies support a dose-response relationship.

6. Bio plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including colorectal and rectal cancer. It was discovered that the digestive tissues (small and large bowel) in Patas monkeys are sensitive targets for NDMA-induced DNA adduct damage (e.g. O(6)-methylguanine),¹²¹ which can lead to digestive cancers. This study supports the bio plausibility of NDMA causing colorectal and rectal cancer.

There are also human tissue and cell studies that support the bio plausibility of intestinal cancer. For example, NDMA was generated in a dynamic *in vitro* human gastrointestinal model, in which gastric conditions closely simulate the physiology observed in humans²⁸³. In the human colon carcinoma cell line (Caco-2), NDMA stimulated oxidative stress, inflammation, and a pro-inflammatory immune response leading to increased cancer risk²⁸⁴. Human colon cells (e.g. epithelial cells) can activate NDMA into metabolites that bind to NDMA²⁸⁵. Thus, the enzyme systems that are capable of metabolizing NDMA in cancer causing metabolites are present in the human colon epithelium²⁸⁵. Additionally, an isomer of NDMA which is chemically similar to NDMA, called azoxymethane (AOM), is frequently used world-wide to initiate colon cancer in animals⁷³. Azoxymethane (AOM), also produces the same DNA adducts so this further evidence that NDMA can initiate colon cancer.

Given that NDMA has 9 out of the 10 key characteristics of a carcinogen, and animal tissue studies and human cell studies demonstrate NDMA induced damage of the intestine, this factor weighs heavily in favor of causal association, and I assigned significant weight to this factor in my analysis.

7. Coherence: NDMA-induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large

mammals. Cytochrome P450 enzymes which metabolize NDMA into a carcinogenic metabolite are expressed in the human intestines²⁸⁶. Thus, it makes biological sense that the intestines would have an increased risk of cancer development after ingestion of NDMA, such as from contaminated valsartan. The presence of cytochrome P450 enzymes in the human intestine and animal studies showing the metabolization of NDMA by the intestine is consistent with the biology of cancer and supports a causal association. As such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trial (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the human dietary studies, NDMA animal cancer studies, animal tissue and cell studies, human cell studies, that NDMA in the valsartan-containing drugs increases the risk of and causes colorectal cancer.

Cancer Type #6 - NDMA Causes Pancreatic Cancer

1. Strength of association / Statistical significance.

The Hidajat et al. study (2019) previously discussed in detail above found that pancreatic cancer was the cause of death for 328 study subjects²²⁶. Association was observed as the men in the second, third, and fourth quartiles had increased relative risks of dying from pancreatic cancer (SHR 1.59, 95% CI:1.18-2.15, SHR 2.19, 95% CI: 1.60-3.00 and SHR 2.6, 95% CI: 1.94-3.49, respectively) compared with men in the first quartile (lowest NDMA exposure) with a P-value for trend of 0.42.

I found one dietary study that quantified the amounts NDMA and NDEA exposure and examined the incidence of pancreatic cancer. In Zheng which used a multivariable-adjusted model, a higher risk of pancreatic cancer was observed for the highest dietary intake of NDEA from plant sources (OR of Q4 vs. Q1=1.93, 95% CI: 1.44–2.60, P trend < 0.0001) and from animal sources (OR of Q4 vs. Q1=1.35, 95% CI: 1.03–1.78, P trend,= 0.004), as well as total NDEA from both animal and plant sources (OR Q4 vs. Q1 = 2.28, 95% CI: 1.71–3.04, P trend < 0.0001)²³⁴. Notably, a positive association was detected for NDMA from plant sources (OR Q4 vs. Q1=1.93, 95% CI: 1.42–2.61, P trend < 0.0001) but not for NDMA from animal sources.

I placed moderate weight on this factor as a well-designed human occupational study, Hidajat, reported a statistically significant association and the Zheng dietary study supported a causal relationship between cancer and increased exposure to NDMA from plant sources only (not animal sources) and NDEA (N-Nitrosamine in the same family of chemicals).

2. Consistency of the association: There is both a NDMA occupational study and NDMA dietary study that find a statistically significant causal relationship between increasing NDMA exposure and pancreatic cancer. A search of the animal studies revealed that NDMA has not been utilized to induce pancreatic cancer, which is usually accomplished by direct orthotopic tumor

implantation (implantation of tumor cells directly into the pancreas) and the early animal studies did not focus on NDMA and experimental pancreatic cancer.

The consistency of the association between NDMA exposure and pancreatic cancer in Hidajat and Zheng (plant sources only, not animal) provide support in favor of a causal association, and I placed moderate weight on this factor when assessing causal relationship.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criteria is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question does NDMA cause human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this factor is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from pancreatic cancer²²⁶. NDMA occupational exposure was defined for quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of pancreatic cancer increased: Quartile II 1.59 (1.18, 2.15), Quartile III 2.19 (1.60, 3.00), and Quartile IV 2.6 (1.94, 3.49) with a P-value for trend of 0.42.

I placed moderate weight on this aspect of causality as the well-designed Hidajat study and Zheng study (plant sources only) support a dose-response relationship.

6. Bio Plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including pancreatic cancer. The animal studies support the bio plausibility of NDMA causing pancreatic cancer: For example, NDMA was carcinogenic in an *in vitro* model of human pancreatic cancer carcinogenesis and induced proliferation²⁸⁷. This human cancer tissue developed tumors within eight weeks after injection into the mice²⁸⁷. NDMA also induced pro-mutagenic DNA adducts (e.g. O6-methylguanine) in the monkey pancreas¹²¹, supporting the bio plausibility of NDMA causing pancreatic cancer.

Human pancreatic cells derived from NDMA-treated human pancreatic cancer explants produced multiple nodules of carcinoma when injected subcutaneously into nude mice^{287,288}. An explant is a technique in this case to grow human pancreatic tissues outside a person and manipulate the tissue by treating the human tissue with a carcinogen such as NDMA. When these NDMA-treated pancreatic cells were injected into mice, they caused tumor growth (See Fig. 3 below).



Figure 3. Tumor growth in a mouse 16 weeks after injecting the animal with cells from human pancreatic cells that were grown in culture for 12 weeks in the presence of NDMA.

The formation of the DNA adduct O6-methylguanine can be detected in human pancreatic explants incubated *in vitro* with NDMA²⁸⁹. NDMA induced both ductal hyperplasia and atypia of the epithelial linings of main ducts, smaller ducts, and ductules within 6 weeks, and carcinoma by the tenth week of human pancreas cultures. Importantly, cells derived from 10-week-old NDMA-treated explants produced multiple nodules of carcinoma when injected subcutaneously into nude mice²⁸⁸. The pancreas tissue in Patas monkeys is a sensitive target for NDMA-induced DNA adduct damage (e.g. O6-methylguanine),¹²¹ which can lead to pancreatic cancer.

Given that NDMA has 9 out of the 10 key characteristics of a carcinogen, and animal tissue and human tissue studies demonstrate NDMA induced damage of the pancreas, this factor weighs heavily in favor of causal association, and I assigned significant weight to this factor in my analysis.

7. Coherence: NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all blood tissues throughout the body. Cytochrome P450 enzymes which metabolize NDMA into its carcinogenic metabolite are expressed in the human pancreas^{290,291}. Thus, it makes biological sense that the pancreas would have an increased risk for cancer development after ingestion of NDMA, such as from contaminated valsartan. The presence of cytochrome P450 enzymes in the human pancreas, and human tissue and animal studies showing the metabolization of NDMA by the pancreas are consistent with the biology of cancer and support a causal association. As such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding

of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the Hidajat study, animal tissue studies, human tissue studies, that NDMA in the valsartan containing drugs increases the risk of and causes pancreatic cancer.

Cancer Type #7 - NDMA Causes Esophageal Cancers

1. **Strength of association / Statistical significance.** The Hidajat et al. study (2019) previously discussed in detail above found that esophageal cancer was the cause of death for 333 study subjects²²⁶. A significant strength of association was observed as the men in the second, third, and fourth quartiles had increasing risks of dying from esophageal cancer (SHR 1.7, 95% CI: 1.24-2.33, SHR 2.43, 95% CI:1.78-3.31, and SHR 3.04, 95% CI:2.26-4.09, respectively) compared with men in the first quartile (lowest NDMA exposure) with a P-value for trend of 0.26.

A couple of dietary studies quantified the amounts of NDMA exposure and examined the incidence of esophageal cancer. Loh et al. found a non-statistically significant increase of esophageal (HR 1.13, 95% CI: 0.77 -1.68). Rogers et. al. found a statistically significant increase for the risk of all oral cancers in the highest exposure category (OR 1.82, CI: 95%: 1.10- 3.00; P-trend 0.118) but showed a non-statistically significant increased risk for larynx (OR 1.70, CI: 95%:

0.91-3.18; P-trend 0.258) and esophageal (OR 1.86, CI: 95%: 0.87-3.95; P-trend 0.063) for the highest exposed group. A statistically significant 15% increased risk of esophageal squamous cell carcinoma was reported in the Keszei et al. study of more than 120,000 people (HR 1.15, 95% CI: 1.05-1.25; P-trend=0.01 based on the tertiles in intake)²³².

Weighing the available studies, I placed significant weight on this factor as the well-designed Hidajat study reported a statistically significant increased risk of death and Keszei dietary study found increased risk of esophageal cancer with statistical significance while other studies found non-statistically significant increased risks.

2. **Consistency of the association:** Hidajat reported an increased risk of death associated with esophageal cancer and one dietary study find a statistically significant causal relationship between increasing NDMA exposure and esophageal cancer. I also examined the consistency of the association between NDMA exposure and cancer across mammalian species. There were no NDMA animal studies that reported the development of esophageal cancer as NDEA preferentially induces esophageal cancer. The consistency of the association between the occupational study and dietary study gives moderate support in favor of a causal association.

3. **Specificity of the association:** Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. **Temporality:** This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA

must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this factor is satisfied.

5. **Biologic gradient/Dose-Response:** In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from esophageal cancer²²⁶. NDMA occupational exposure was defined for quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of esophageal cancer increased: Quartile II 1.7 (1.24, 2.33), Quartile III 2.43 (1.78, 3.31), and Quartile IV 3.04 (2.26, 4.09) with a P-value for trend of 0.26.

Rogers et. al. found a statistically significant increase for the risk of all oral cancers in the highest exposure category, (OR 1.82, 95% CI: 1.10-3.00) but a non-statistically increased risk of esophageal cancer (OR 1.86, 95% CI: 0.87-3.95). However, a statistically significant increased risk of 15% for esophageal was found in the Keszei et al. study of more than 120,000 people. (HR 1.15, 95% CI: 1.05-1.25 men); (HR 1.34, 95% CI: 1.04-1.71 women).

I placed significant weight on this aspect of causality as the Hidajat reported an increased risk of death with increasing exposures and Keszei studies support a statistically significant dose-response relationship and Rogers showed an increased risk even though not statistically significant.

6. **Bio plausibility:**

As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including esophageal cancer. In particular, there is a study that supports the bio plausibility of NDMA causing esophageal cancer as evidence of human esophageal tissues activating NDMA via cytochrome P450s 3A4 and 2E1²⁹². Human esophageal tissue can metabolize NDMA into electrophilic metabolites that bind to

DNA¹³⁰. NDMA was metabolized to both CO₂ and carbonium ions¹³⁰. The NDMA-induced carbonium ions causes methylation of DNA bases including O6-methylguanine, 3-methyladenine, and 7-methylguanine¹³⁰. A follow-up study confirmed that the metabolism of NDMA and NDEA as measured by the alkylation of DNA was similar in both rat and human esophagus²⁹³. As measured by both CO₂ and formaldehyde, NDMA is metabolized by the human and rat esophagus into the guanine position 7 and O6 of the DNA²⁹³. These NDMA-induced adducts are identical to the ones found in human lung and colon. These NDMA-induced adducts and the metabolic pathways leading to them are similar to those found in experimental animals who developed NDMA-induced cancers¹³⁰. Thus we can extrapolate the results of NDMA causing cancer in animals directly to humans¹³⁰. The esophageal tissue in Patas monkeys is another sensitive target for NDMA-induced DNA adduct damage (e.g. O(6)-methylguanine),¹²¹ which can lead to esophageal cancer. The presence of cytochrome P450 enzymes in the human esophagus and animal studies showing the metabolization of NDMA by the esophageal tissues support a causal association. As such, I assigned this factor significant weight.

7. **Coherence:** NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogenesis). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all

blood tissues throughout the body. Cytochrome P450 enzymes that metabolize NDMA into its carcinogenic metabolite are expressed in the human esophagus. Thus, it makes biological sense that the esophagus would have an increased risk of cancer development after ingestion of NDMA, such as from contaminated valsartan. This is consistent with biology of cancer and supports a causal association. As such, I assigned this factor significant weight.

8. **Experiment:** As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. **Analogy:** N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a finding a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the Hidajat and Keszei studies, NDMA human tissue and animal tissue studies, that NDMA in the valsartan containing drugs increases the risk of and causes esophageal cancer.

Cancer Type #8 - NDMA Causes Prostate Cancer

1. **Strength of association / Statistical significance.** The Hidajat et al. study (2019) previously discussed in detail above found that prostate cancer was the cause of death for 885 study subjects²²⁶. A significant strength of association was observed as the men in the second,

third, and fourth quartiles had increasing statistically significant relative risks of dying from prostate cancer (SHR 2.32, 95% CI: 1.82-2.97, SHR 4.87, 95% CI: 3.89-6.11, and SHR 5.36, 95% CI: 4.27-6.73 respectively) compared with men in the first quartile (lowest NDMA exposure) with a P-value for trend of less than 0.01.

A few dietary studies quantified the amounts of NDMA exposure and examined the incidence of prostate cancer. Loh et al. found no statistically increased risk of prostate cancer. Jakszyn found an increased risk of 23% for localized prostate cancer (HR 1.23, 95% CI: 0.99-1.53) that nearly reached statistical significance for localized prostate cancer only.

I placed moderate weight on this factor as a well-designed human epidemiological study (Hidajat) found increased risk with statistical significance, one dietary study found no increased risk (Loh) but another dietary study (Jakszyn) found an increased risk of localized prostate cancer that did not rise to statistical significance.

2. Consistency of the association.

There are NDMA occupational and dietary studies that find a statistically significant causal relationship between increasing NDMA exposure and prostate cancer. I was unable to identify any animal model studies that were designed to study the causal relationship between NDMA and prostate cancer. However, Hidajat (occupational study) found a statistically significant increased risk, Loh found no statistically significant increased risk, and Jakszyn found a 23% non-statistically significant risk, so I assigned moderate weight to this factor in my causal analysis.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood, and lung. As such, this

Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this criterion is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death related to prostate cancer. NDMA occupational exposure was defined for quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of prostate cancer increased: Quartile II 2.32 (1.82, 2.97) Quartile III 4.87 (3.89, 6.11), and Quartile IV 5.36 (4.27, 6.73) with a P-value for trend of less than 0.01. Loh et al. found no increased risk of prostate cancer. Jakszyn studied prostate cancer risk and found a non-statistically significant increased risk of 23% (RR 1.23, 95% CI: 0.99-1.53) for localized prostate cancer only.

I placed moderate weight on this aspect of causality as the Hidajat study, an occupational study with a sound design, strongly supports a dose-response relationship while Loh found no increased risk but Jakszyn found a non-statistically significant increased risk.

6. Bio plausibility: As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including prostate cancer. I did not locate any animal bioassays designed to study the relationship between NDMA and prostate cancer. However, the prostate tissue in Patas monkeys is a sensitive target for NDMA-induced DNA adduct damage (e.g. O6-methylguanine),¹²¹ which can lead to prostate cancer. The

human tissue and animal tissue studies showing the metabolism of NDMA by the prostate supports a causal association. As such, I assigned this factor significant weight.

7. Coherence: NDMA-induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogenesis). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all blood tissues throughout the body. Cytochrome P450 enzymes that metabolize NDMA into its carcinogenic metabolite are expressed in the human prostate²⁹⁴. Thus, it makes biological sense that the prostate would have an increased risk for cancer development after ingestion of NDMA, such as from contaminated valsartan. I assigned this factor moderate weight in my analysis.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely

high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the NDMA human occupational and dietary studies, that NDMA in the valsartan containing drugs increases the risk of and causes prostate cancer.

Cancer Type #9 - NDMA Causes Lung Cancer

1. Strength of association / Statistical significance. The Hidajat et al. study (2019) previously discussed in detail above found that lung cancer was the cause of death for 3377 study subjects²²⁶. A significant association was observed as the men in the second, third, and fourth quartiles had increased relative risks of dying from lung cancer (SHR 1.21, 95% CI:1.10-1.32; SHR 1.54 95% CI: 1.39-1.70; and SHR 1.7, 95% CI: 1.54-1.87, respectively) compared with men in the first quartile (lowest NDMA exposure) with a P-value for trend of 0.36.

A few dietary studies quantified the amount NDMA exposure and also examined the incidence of lung cancer. De Stefani studied NDMA associated lung cancer risk and found a statistically significant increased risk (OR 3.14, CI: 95%, 1.86-5.29) as did Goodman for high nitrosamine diets in men (Men OR Q4 v. Q1=3.3, CI: 95%, 1.7-6.2). The values reported in women also showed increased risk that did not reach statistical significance (Women OR Q4 v Q1=2.7, CI: 95%, 1.0-6.9). Loh found an increased risk that was not statistically significant. (HR 1.05, CI:95%, 0.88-1.24).

I placed significant weight on this factor as a well-designed human epidemiological study and two dietary studies found increased risk with statistical significance.

2. Consistency of the association.

There are NDMA occupational and dietary studies that find a statistically significant causal relationship between increasing NDMA exposure and lung cancer. I also examined the consistency of the association between NDMA exposure and cancer across mammalian species. NDMA has been shown to be a powerful carcinogen by many scientists in rat and mouse studies.

For example in Magee and Barnes (1962), NDMA in the diet induced large kidney, liver, and lung tumors *after 26 weeks* in 13 of 18 rats⁹⁰. In Zak et al (1959), NDMA induced lung tumors in rats between *111 and 160 days*²⁹⁵. Takayama and Oota reported lung tumor induction (adenomas and adenocarcinomas) in mice after feeding them a diet containing NDMA²⁵². Clapp and Toya 1970 observed lung tumors developed in mice that were exposed to NDMA daily via drinking water⁹⁷. Otsuka and Kuwahara (1971) demonstrated that daily administration of NDMA in the diet also induced lung tumors in mice⁸¹.

NDMA induced lung adenomas in Balb/c mice, a strain which has low spontaneous incidence of tumors⁹⁸. Lung adenomas were found in all three strains of mice (ddN, ICR, and C3H) when different levels of NDMA were given in the diet²⁵³. Toth *et al. showed* a single subcutaneous dose of NDMA to adult and newborn BALB/c mice induced high incidences of lung adenomas²⁴⁹. NDMA induced 99% of lung tumors as compared with control values of 37% after oral administration in drinking water⁹⁶. NDMA induced lung adenomas in nearly 100% of mice after oral administration⁹⁷.

In Anderson et al. (1986), NDMA induced lung tumors by *16 to 28 weeks* in mice²⁴⁸. In Toth et al. (1964), a single injection of NDMA induced lung adenomas *after 141 days* in mice²⁴⁹. In Otsuka et al. (1971) NDMA in the diet induced lung tumors (e.g. in 89/115 mice) *after 5 months* in mice⁸¹. A single dose of NDMA given to mice at 60 days of age induced a large increase in the incidence of lung tumors (76% - 100% compared with 19-25% in controls)²⁹⁶. Oral exposure to

NDMA also yielded lung tumors in mice⁵. Pulmonary tumors developed in male and female rats fed a diet containing NDMA³⁹.

Thrice weekly intraperitoneal injection of male C3H mice with NDMA for seven weeks resulted in 37 of 38 mice with lung tumors, compared to 7 of 28 in animals which were not exposed to NDMA¹¹⁸. A single dose of NDMA of 5, 10, or 15 mg/kg induced 9/18, 16/19, and 4/5 lung tumors (adenomas and papillary carcinomas) in male RF mice as compared with 25/52 in controls²⁹⁷. The tumorigenic effect of NDMA given once at 5 different dose levels was compared in adult Swiss, ASW/SN and A-strain mice. NDMA at the highest dose level given to Swiss and ASW/SN mice increased for lung neoplasms²⁹⁸. A single subcutaneous injection of NDMA was administered to Swiss mice at 5 dose levels. A statistical increase in the incidence of lung tumors was observed at all dose levels of NDMA except for the group receiving the lowest dose of NDMA²⁹⁹. After a single subcutaneous injection of NDMA, a dose-response relationship was seen for lung tumors (adenomas and carcinomas).

Balb/c mice and Wistar rats were exposed to daily inhalation of NDMA for 17 months and 25 months developed lung compared to control animals⁷⁸. Pulmonary neoplasms developed in rats on a diet containing NDMA²⁹⁵. NDMA can induce lung adenocarcinoma and squamous cell carcinoma^{295,300}. In larger animals such as rabbits, Le Page et al (1969) demonstrated that NDMA induced metastases in the lung⁹¹.

The consistency of the association between NDMA exposure and lung cancer across the Hidajat occupational study, dietary studies, and animal studies, studied by several researchers over decades, using different study designs, is significant and gives strong support in favor of a causal association.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreas, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criterion is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this factor is satisfied.

5. Biologic gradient/Dose-Response: In Hidajat, a strong dose response was shown as the men with increased occupational exposure to NDMA had increasing incidents of death from lung cancer. NDMA occupational exposure was defined for Quartile I as less than 3.12 year $\mu\text{g}/\text{m}^3$, Quartile II as 3.12 - 5.96 year $\mu\text{g}/\text{m}^3$, Quartile III as 5.96 – 9.67 year $\mu\text{g}/\text{m}^3$, and Quartile IV as greater than 9.67 year $\mu\text{g}/\text{m}^3$. With increasing exposure, the incidence of lung cancer increased: Quartile II 1.21 (1.10, 1.32), Quartile III 1.54 (1.39, 1.70), and Quartile IV 1.7 (1.54, 1.87) with a P-value for trend of 0.36. De Stefani studied lung cancer risk and found a statistically significant increased risk (OR 3.14, 95% CI: 1.86-5.29) that increased with NDMA exposure dose. Goodman also reported an increasing risk as the exposure to NDMA increased for Quartile 4 v. Quartile 1 (Men OR=3.3, 95% CI: 1.7-6.2) (Women OR=2.7, 95% CI: 1.0-6.9) reporting increasing risk as the exposure to NDMA increased.

I placed significant weight on this aspect of causality as the Hidajat study and two dietary studies supported a dose-response relationship.

6. Bio Plausibility:

As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including lung cancer. In particular, the following studies support the bio plausibility of NDMA causing lung cancer.

Human lung tissues (e.g. human bronchi) metabolize NDMA into DNA adducts at the O-6 and N-7 positions of guanine indicating their metabolic activation is the same as in animals⁴⁴. Slices of human lung metabolize NDMA, as measure by the production of CO₂, at a rate significantly higher than that for the rat lung³⁰¹. Thus, human lung and liver can metabolize NDMA to reactive cancer-causing metabolites via the same metabolic pathway as in animal species^{44,125,301}. A fraction of the dose may be eliminated in the expired air²¹⁴. The lung tissue in Patas monkeys is a sensitive target for NDMA-induced DNA adduct damage (e.g. O(6)-methylguanine),¹²¹ which can lead to lung cancer. The metabolization of NDMA by human and animal lung tissue support bio plausibility and a causal association. As such, I assigned this factor significant weight.

7. Coherence: NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogens). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all

blood tissues throughout the body. Cytochrome P450 enzymes that metabolize NDMA into its carcinogenic metabolite are expressed in the human lungs³⁰². Thus, it makes biological sense that the lungs would have an increased risk for cancer development after ingestion of NDMA, such as from contaminated valsartan. The presence of cytochrome P450 enzymes in the human lung showing the metabolism of NDMA by the lung is consistent with biology of cancer and supports a causal association. As such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs), so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human *in vitro* studies provide significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. This lends support to a causal relationship between NDMA exposure and human cancer. I assigned moderate weight to this factor.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the Hidajat occupational study, two dietary studies, and animal cancer studies, that NDMA in the valsartan containing drugs increases the risk of and causes lung cancer.

Cancer Type #10 - NDMA Causes Kidney Cancer

1. Strength of association / Statistical significance.

The Hidajat study did not evaluate the causal relationship between kidney cancer and NDMA exposure. In my review of the dietary studies that quantified the amount of NDMA and/or NDEA exposure, I did not find any that studied kidney cancer.

The animal cancer studies, however, showed consistent evidence across multiple species of kidney cancer caused by exposure to NDMA. NDMA caused kidney tumors in rats and mice exposed orally or by inhalation or intraperitoneal injection and in rats exposed prenatally or by subcutaneous injection. Here are a few examples: Magee and Barnes (1962) showed of rats that survived a single dose of NDMA 20-30% of them developed NDMA-induced kidney tumors. In addition, NDMA in the diet (for 1 to 4 weeks) induced large kidney tumors *after 26 weeks* in 13 of 18 rats⁹⁰. The resemblance between the NDMA-induced kidney tumors induced by NDMA in the rat and the kidney tumors in humans was very close⁹⁰. In rats, short-term NDMA or single-dose produces kidney tumors^{90,295,303-305}. A single subcutaneous injection of NDMA to adult and newborn BALB/c mice induced adenomas of kidney²⁴⁹. NDMA also induced developed kidney adenomas⁸³ in another study. A single dose of NDMA given to mice at 60 days of age induced large and increased kidney adenomas and carcinomas in males compared to controls²⁹⁶. In Terracini et al (1964) in a high profile publication in *Nature*, a single administration of NDMA induced kidney tumors in newborn and 1-week old rats⁷⁵. NDMA induced very large kidney tumors (up to 8 cm), in rats *by 36 weeks* of age that were all of the very aggressive anaplastic type⁷⁵. No renal or liver tumors were observed in untreated rats⁷⁵. In Zak et al (1959), NDMA induced kidney and lung tumors in rats between *111 and 160 days*²⁹⁵. Kidney tumors developed in male and female Sprague-Dawley rats fed a diet of NDMA for around 80 days. The tumors found in the kidneys can be classified into 2 types (1) benign solid and cystic adenomas occurring in rats fed NDMA for less than 160 days (2) anaplastic epithelial tumors in animals treated longer than 160 days²⁹⁵.

Oral administration of NDMA induced papillary cystadenomas of the kidney in 5 cases with malignant tumors of the kidney in 2 cases in mice²⁵³. NDMA induces kidney cancer in mice after oral administration. In three strains of mice (ddN, ICR, and C3H) when different levels of NDMA were given in the diet, high incidences of kidney cystadenomas were found²⁵³. In Swan et al (1980), NDMA induced kidney tumors in rats by *28-43 weeks*³⁰⁶. In studies by Mohr et al (1974) in hamsters, NDMA induced kidney tumors after *13 to 28 weeks*²⁴⁷. In Toth et al (1964), a single injection of NDMA induced adenomas of the kidney *after 141 days* in mice²⁴⁹. In Otsuka et al (1971) NDMA in the diet induced kidney tumors *after 5 months* in mice⁸¹.

Kidney tumors developed in male and female rats fed a diet containing NDMA for more than 80 days³⁹. Epithelial tumors (8.6%) and mesenchymal tumors (14.5%) developed in the kidneys of rats following treatment with NDMA for 6 days^{307,308}. Rats subcutaneously administered NDMA on day 1, 7, 21 or 70 resulted in significant increases in kidney tumors⁸⁷. Single injections of NDMA at day 21 or 70 after birth induced 38% kidney tumors after median latent period of 286-369 days; 41% of kidney tumors were of the renal cell type and 59% were stromal nephromas. No control rats developed kidney tumors⁸⁷. Abdominal palpation was highly accurate for relatively early diagnosis of renal problems. NDMA doses below the LD50 induced kidney tumors in every rat.

A single dose of NDMA led to the development of kidney cancers in all the surviving rats (14/14) after 8-11 months compared with 0 of 9 control rats³⁰⁹. Tumors of the kidney developed in 33% of male and 63% of female Wistar rats that received a single intraperitoneal dose of NDMA³¹⁰. Morphologically the tumors were of epithelial and mesenchymal type with more of the epithelial type³¹⁰. A single injection of NDMA to Wistar rats induced kidney tumors³¹¹. The histology of NDMA-induced kidney tumors has been described^{312,313}. Subcutaneous injections to

Wistar rats of NDMA, beginning at birth, induced kidney tumors in 6 of 11 rats, mainly nephroblastomas, adenomas, and clear-cell carcinomas³¹⁴. A single administration of NDMA induced tumors of the kidney on different strains³¹⁵. Even in rabbits, Le Page et al (1969) demonstrated that NDMA induced a kidney tumor after 23 weeks⁹¹.

Since there are an overwhelming number of animal studies that demonstrate NDMA causes kidney cancer and but no human epidemiology studies finding statistical significance, I placed moderate weight on this factor in my causation analysis.

2. Consistency of the association.

There are no human epidemiology studies showing a consistency of the association, but NDMA has been shown to be a powerful carcinogen by many scientists across multiple animal species. As set forth in detail above, there are multiple animal studies showing that mice, rats, hamsters and rabbits all develop kidney cancer when exposed to NDMA.

There is a lack of human epidemiology studies showing an association between NDMA and kidney cancer. However, there is a consistency of the association between NDMA exposure and kidney cancer across many mammalian species, studied by several researchers, using different study designs, over several decades is significant and gives moderate support in favor of a causal association.

3. Specificity of the association: Most carcinogens cause more than one type of cancer. Thus, this factor is much less relevant when answering the question of whether NDMA causes human cancer. NDMA has been shown to cause cancers of the liver, kidney, stomach, colorectal/intestine, pancreatic, esophagus, bladder, prostate, blood and lung. As such, this Bradford Hill criterion is less relevant for this cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

4. Temporality: This factor is important in the causal analysis. In any study of scientific value to answer the question of whether NDMA causes human cancer, the exposure to NDMA must come before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDMA came before the diagnosis of cancer so this factor is satisfied.

5. Biologic gradient/Dose-Response: There are no human epidemiology studies that show dose-response for NDMA and kidney cancer. The animal cancer studies show a strong dose-response association between NDMA exposure and kidney cancer. The studies demonstrated an increasing rate of kidney cancer with increasing dose. For example, a single dose of NDMA induces kidney tumors in rats in a sigmoidal dose response curve. The incidence of renal mesenchymal tumors induced in rats by NDMA via a sigmoidal dose-response curve³¹⁶. The evidence in the animal studies demonstrate a classic dose-response between NDMA and kidney cancer. However, there are no human epidemiology studies that show dose-response so I assigned this factor lower weight.

6. Bio Plausibility:

As set forth in detail in the Key Characteristics section of this report, there are 9 clear biologically plausible mechanisms for NDMA to cause cancer including kidney cancer. Importantly, Magee and Barnes demonstrated in 1962 that rats given NDMA in the diet (for 1 to 4 weeks) induced large kidney tumors *after 26 weeks* in 13 of 18 rats⁹⁰. The resemblance between the NDMA-induced kidney tumors induced by NDMA in the rat and the kidney tumors in humans was very close⁹⁰.

The kidney tissue in Patas monkeys is sensitive targets for NDMA induced DNA adduct damage (e.g. O(6)-methylguanine),¹²¹ which can lead to kidney cancer. In particular, these animal bioassays as well as animal and human tissue and cell studies support the bio plausibility of NDMA

causing kidney cancer. Given that NDMA has 9 out of the 10 key characteristics of a carcinogen, this factor weighs heavily in favor of causal association, and I assigned great weight to this factor in my analysis.

7. Coherence: NDMA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDMA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogenesis). In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. NDMA enters the systemic circulation which provides a functional blood supply to all blood tissues throughout the body. Cytochrome P450 enzymes are expressed in the kidneys that can metabolize NDMA into its carcinogenic metabolite so it makes biological sense that the kidneys would have an increased risk for cancer development after ingestion of NDMA, such as taking contaminated valsartan. This supports a causal association and as such, I assigned this factor significant weight.

8. Experiment: As NDMA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDMA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDMA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDMA RCTs in humans, I attribute less weight to this factor, even though non-human models and human in vitro studies provide

significant support.

9. Analogy: N-nitroso compounds, which include NDMA, are known to display extremely high carcinogenic potency. Given that the class of N-nitroso compounds is known to be carcinogenic, this lends support to a finding a causal relationship between NDMA exposure and human cancer including kidney cancer.

Conclusion: It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the NDMA animal cancer studies, animal tissue and cell studies, as well as human tissue and cell studies, that NDMA in the valsartan-containing drugs increases the risk and causes kidney cancer.

NDEA IS A POTENT CARCINOGEN

In September 2018, the FDA announced that in addition to NDMA, another potent carcinogen, N-nitrosodiethylamine (NDEA), was discovered in valsartan containing drugs which prompted more recalls. Using the same methodology as was applied to NDMA, the FDA derived the daily acceptable intake values for NDEA in valsartan from compound-specific animal toxicological data using the TD50 estimate of the lowest dose, which is 95% certain to cause no more than a 10% cancer incidence in rodents, as the point of departure for the calculation of excess cancer risk.

The evaluation of whether NDEA is a human carcinogen follows the same analysis described above in detail for NDMA. NDEA and NDMA both belong to the class of nitrosamines and are classified probable human carcinogens.

The FDA recognized the danger of valsartan tablets containing NDMA and/or NDEA and set strict daily acceptable intake limits for NDMA (0.3 ppm) and NDEA (0.083 ppm) in valsartan. Based on the maximum valsartan dose of 320 mg, this equates to 96 nanograms of NDMA and 26.5 nanograms of NDEA. See FDA Update 2/28/2019, Table of Interim Limits for Nitrosamine

Impurities in ARBs, <https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan>. The

detectable levels of NDMA and NDEA found in the contaminated valsartan tablets are much higher than these values. For example, the batch containing 16.93 ppm of NDEA is 204 times higher than the acceptable intake established by the FDA. The FDA established 26.5 nanogram daily acceptable intake limit of NDEA corresponds to a lifetime cumulative dose of 677 micrograms. Notably, the rat carcinogenic potency of NDEA is about **three times** that of NDMA³¹⁷. This is was the rationale on why the FDA established 26.5 nanogram daily acceptable intake limit of NDEA which is less than the 96 nanogram daily acceptable limit for NDMA. Like NDMA, NDEA is genotoxic³¹⁸. NDEA is carcinogenic in **all** investigated animal species at relatively low dosages³¹⁹. Similar to NDMA, no threshold has been detected for the cancer causing activity of NDEA (e.g., Peto *et al* 1991)^{19,319}.

IARC is the International Agency for Research on Cancer, a scientific group. **IARC** has classified both NDEA and NDMA as “probable human carcinogens.” Similarly, the US Environmental Protection Agency (EPA) and Department of Human Health Services consider both NDMA and NDEA as “human carcinogens”. **IARC** Evaluation, 1978, US EPA Integrated Risk Information System, Chemical Assessment Summary for N-Nitrosodiethylamine, 1987.

The **EPA** is the Environmental Protection Agency, the federal agency responsible for regulating environmental hazards. The EPA has classified NDEA as “B2; probable human carcinogen” based on the induction of tumors at multiple sites in both rodent and nonrodent species exposed by various routes. **Many scientists believe there is no safe level of exposure to a carcinogen.** According to the New Jersey Department of Health, NDEA is a **“PROBABLE CARCINOGEN in humans. There may be no safe level of exposure to a carcinogen, so all contact should be reduced to the lowest possible level. “**

NTP is the National Toxicology Program in the United States which tests chemicals and reviews evidence for cancer. The NTP Report on Carcinogen is a "...congressionally mandated, science-based, public health report that provides a cumulative list of known or reasonably anticipated human carcinogens." These reports are prepared by NTP for the US Department of Health and Human Services.

In its Report on Carcinogens, Fourteenth Edition (November 2016) discussing NDEA, NTP found that NDEA, "*...is reasonably anticipated to be a human carcinogen from studies in experimental animals*". Because NDEA is so well known to be a potent carcinogen in animal experimentation, there is a dearth of human epidemiological studies evaluating human exposure for obvious ethical issues. There has been one dietary study, Zheng *et al.*, that quantified NDMA and NDEA exposure, and risk of human pancreatic cancer²³⁴. The study reported 89% statistically significant increase in risk of pancreatic cancer and is discussed in more detail below. All investigating official organizations and agencies have identified NDEA as either "probable" or "reasonably anticipated" to be a human carcinogen.

N-Nitrosodiethylamine (NDEA) - Background

According to the NTP Report on Carcinogens, Fourteenth Edition, NDEA is available in small quantities for research purposes, no evidence was found that it was manufactured commercially. NDEA is a pale yellow, volatile liquid with an *Amine* or *Aromatic* odor at room temperature. It was previously used as an additive to gasoline and lubricants, as a stabilizer in plastics, and in research. NDEA is found in cigarettes and cigarette smoke at 0.0083–0.0405 ug/cigarette, as well as in low concentrations in rolls, buns, muffins, millet flour, bagels, ham, salami, dried cuttlefish and even oysters (0.109 ug/100 g)^{320,321}.

NDEA is used to *cause* cancer in animal models to study the molecular mechanisms and chemoprevention of various anti-cancer drugs³²². NDEA should be handled with extreme caution

as a carcinogen and mutagen. As a testament to how potent a carcinogen NDEA is in causing cancer, liver cancer was initiated by a *single* dose of NDEA in over 17 publications³²³⁻³³⁹. Accordingly, NDEA is used as a gold standard world-wide in over 40 scientific peer-reviewed publications to initiate and cause cancer in various animal species such as rats and mice^{322-328,337-370}. Similar to NDMA, NDEA causes tumors in every animal species tested²⁰².

In addition, NDEA induces different tumor types in various organs including liver, esophagus, and kidney^{74,222,225,326,327,352,371-375}. According to the above **NTP** Report on Carcinogens, NDEA caused tumors in rabbits after subcutaneous injection (Huntrakoo, et.al, 1989 NTP Report on Carcinogen); snakes after oral exposure including kidney, liver, oral cavity and trachea (Schmahl and Scherf 1983, 1984)^{376,377} the addition of NDEA to tank water increased the incidence of malignant pancreatic tumors in larval or juvenile fish (Thiyagarajah and Grizzle 1986,^{378,379}) and tumors of the digestive gland and hematopoietic (blood) system in mollusks (Khudoley and Syrenkko 1978 NTP Report on Carcinogens). Laryngotracheal tumors were observed in pregnant hamsters exposed via injection and the prenatally exposed offspring developed laryngotracheal tumors (neuroendocrine-cell tumors) in the second generation of offspring (Mohr, et.al, 1995, 1989 NTP Report on Carcinogen). The **NTP** Report on Carcinogens, Edition for NDEA also describes studies showing liver tumors in chickens after intramuscular administration and cats after oral administration (dietary or stomach tube) (Schmahl et.al, 1978, NTP Report on Carcinogens).

As shown above, NDEA is carcinogenic in all animal species tested including mice, rats, Syrian golden, Chinese and European hamsters, guinea pigs, rabbits, dogs, gerbils, pigs, chickens, cats, monkeys, hedgehogs, various fish, frogs, and birds. NDEA induces benign and malignant tumors after its administration by every route of transmission, including ingestion, parenteral administration, inhalation, and rectal installation. The major target organs are the liver, respiratory,

and upper digestive tracts and kidney. NDEA is carcinogenic following its administration prenatally and in single doses. In several studies, dose-response relationships were established.

IARC (1978) reported after oral NDEA exposure, mice developed liver, esophageal, and forestomach tumors; rats developed liver, esophageal and kidney tumors; Syrian golden hamsters developed tumors of the trachea, lung, liver, nasal cavity, and bronchi; Chinese hamsters developed forestomach, esophageal and liver tumors; guinea pigs and rabbits had liver tumors; dogs had liver and nasal cavity tumors; pigs had liver, kidney and brain tumors; and monkeys had liver tumors²⁰². IARC concluded “there is *sufficient evidence* of a carcinogenic effect of *N*-nitrosodiethylamine in many experimental animal species. Although no epidemiological data were available (by 1978), *N*-nitrosodiethylamine (NDEA) should be regarded for practical purposes as if it were carcinogenic to humans.”

Between 1961 and 1967 many cancer studies demonstrated that NDEA caused liver, gastrointestinal, skin, respiratory and hematopoietic tumors in mice; rats developed primarily liver (e.g. hepatocellular) and esophageal tumors^{202,218} as well as tumors of the forestomach, tongue, kidney and oropharynx²²²; NDEA caused liver, gastrointestinal and respiratory tumors in guinea pigs and hamsters; rabbits, dogs and monkeys developed liver and nasal cavity tumors; and pigs developed both liver, kidney, brain and hematopoietic tumors²⁰².

Gray et al. (1991) demonstrated that NDEA induced liver, esophageal and stomach tumors in mice³⁸⁰. In the mouse liver, there were both Kupffer cell and hepatocellular tumors, compared with the Colworth rats evaluated by Peto et al. (1991a), whose livers developed hepatocellular tumors¹⁹. Hyperplasia was noted along with the hepatocellular and esophageal tumors. In hamsters exposed to NDEA by Gray et al. (1991), there were liver-cell and bile-duct tumors³⁸⁰. NDEA-exposed monkeys also developed hepatocellular carcinomas²²⁴. Hepatic cell carcinoma in monkeys was induced within 27 months (earliest tumor 14 months, median time to tumor

formation, 23 months) in 5 of 11 macaque monkeys including metastases to the lung⁵⁰. Thus, NDEA induces cancer in both rodents and primates, as well as all other species studied.

NDEA is a Mutagen and Carcinogen

A **carcinogen** is a substance that causes cancer. Carcinogens are classified according to their mode of action as genotoxic or nongenotoxic carcinogens. A **mutagen** is a substance that causes mutations. A **mutation** is a change in the genetic material in a body cell. Mutations can lead to birth defects, miscarriages, or cancer. Compounds that predispose cells to develop tumors, either by direct genotoxicity, or through indirect, non-genotoxic pathways, are called initiators and the normally non-DNA reactive compounds that stimulate tumor development are called promoters²¹. Approximately 70% of known mutagens are also carcinogens or cancer-causing compounds³⁸¹. A compound that acts as both an initiator and a promoter is referred to as a '**complete carcinogen**' because tumor development can occur without the application of another compound, i.e., a promoter³⁸². NDEA, like NDMA, is a complete carcinogen.

The Ten Key Characteristics of Carcinogens: NDEA

As explained previously for NDMA, there are 10 key characteristics of human carcinogens that provide the basis for an objective approach to identifying and categorizing cancer mechanisms when assessing whether a chemical is a potential human carcinogen^{24,25}. Since the properties of each key characteristic have been detailed in the discussion on NDMA, they will not be repeated here for NDEA as the characteristics are the same.

This systematic approach assists in evaluating chemicals and pharmaceutical agents as potential human carcinogens, especially in the absence of convincing epidemiological data on many human cancers²⁴. The key characteristics of carcinogens described by Smith et al. (2016)^{24,25} which are utilized by **IARC** are as follows:

1. Can the agent act as an electrophilic or can be metabolically activated to an electrophile?
2. Is the agent genotoxic?
3. Does the agent alter DNA repair or causes genomic instability?
4. Does the agent induce epigenetic alterations?
5. Does the agent induces oxidative stress?
6. Does the agent induce chronic inflammation?
7. Is the agent immunosuppressive?
8. Does the agent modulate receptor-mediated effects?
9. Does the agent cause immortalization?
10. Does the agent alter cell proliferation, cell death, or nutrient supply?

The first four characteristics focus on the genotoxic nature of the chemical agent. The last six characteristics focus on the non-genotoxic mechanisms in the tumor microenvironment. These 10 key characteristics of human carcinogens are a roadmap for understanding cancer mechanisms when asking whether a chemical or agent is a potential human carcinogen²⁴.

Similar to my analysis of NDMA, I used these key characteristics to determine the bio plausibility of the mechanisms of action for NDEA-induced carcinogenesis. The range of evidence available to determine whether NDEA exhibits these 10 key characteristics includes the following: animal cancer bioassays, studies of specific biological mechanisms in tissues and cells derived from humans, studies of specific biological mechanisms in tissues and cells derived from animals. I only found one study of exposure to humans in which the amount of NDEA was quantified in the diet. As with NDMA, it would be unethical to perform any human trials with NDEA because it is a human carcinogen.

As a demonstration of its potency, NDEA exhibits 9 of 10 key characteristics of human carcinogens. Following each heading, I have listed those studies that are relevant to NDEA for each key characteristic.

Key characteristic #1 – NDEA is metabolically activated to electrophiles via the formation of DNA adducts.

Similar to NDMA, NDEA is metabolically activated before it causes its potent cancer causing activity²⁰². The rate of metabolism of NDEA by slices of organs from rats and hamsters *in vitro* has been measured, and a correlation has been made between the degree of metabolism and the distribution of NDEA-induced tumors^{125,202}. Metabolic activation of NDEA results in the formation of DNA adducts which react with the DNA as part of the cancer initiation process. Metabolic activation of NDEA forms reactive metabolites resulting in ethylation of liver RNA and DNA to form 7-ethylguanine.

NDEA is activated to electrophiles that react in DNA³⁸³. An electrophile (“electron loving”) is a molecule that accepts electrons to form a bond with its reaction partner. Electrophiles rapidly bind to tissues and can lead to cancer; the electrophilic reactivity correlates with the cancer-causing potency of carcinogens such as NDEA and NDMA. NDEA causes cancer because in the human body it triggers potent electrophilic alkylating agents such as DNA adducts¹⁰⁷. These cancer promoting substances are formed by metabolic activation. These electrophiles react with the DNA of target tissue to form altered bases which leads to the initiation of cancer¹⁰⁷.

Similar to NDMA, NDEA is activated by cytochrome P450 enzymes. Metabolic biotransformation of NDEA by cytochrome P450 enzymes produces DNA adducts such as O6-ethyldeoxyguanosine as well as O4- and O6-ethyldeoxythymidine, and active ethyl radical

metabolites that initiate carcinogenesis³⁸³. Similar to NDMA, NDEA-induced DNA adducts, which if not repaired, can lead to cancer formation³⁸⁴. Once bioactivated, NDEA can attack all four DNA bases at positions with high electron density²⁰². This NDEA-induced alkylation attack in each organ reflects the local level of bioactivation capacity, since the diazonium ion is too reactive to be transported to other organs in significant amounts.

It is shown that NDEA forms ethyl adducts at many DNA positions³⁸⁵. Ethyl adducts at thymine 04 are both persistent and mutagenic²⁰². Ethyl-DNA adducts are formed after *in vivo* exposure to NDEA and their persistence contributes to tumor induction. Since this is a relatively simple and rapid alkylation mechanism, NDEA may produce DNA adducts in *any* tissue with appropriate activating cytochrome P450 enzymes.

NDEA-induced metabolic activation has been demonstrated *in vitro* (outside the body) and *in vivo* (inside the body). This NDEA metabolic activation correlates with the induction of NDEA-induced tumors. After administration of NDEA to rats or hamsters, several ethylated DNA adducts were generated in the liver and kidney DNA. These included 7-ethylguanine, O6 ethylguanine, and 3-ethyladenine^{116,386}. In addition to DNA adducts, there is a correlation between carbon dioxide (CO₂), another tumor promoting metabolite produced from NDEA, in the liver, lung and the organ distribution of NDEA-induced tumors as observed *in vivo*³⁸⁷.

Both NDEA and NDMA cause cancer in tissues remote from the site of administration¹⁰⁷. The first bioactivation step for NDEA- DNA adduct formation in rat liver involves o-hydroxylation of NDEA by CYP2E1 and other P450 isozymes leading to the reactive ethyl diazonium ion²⁰². The metabolic activation of NDEA can be mediated by CYP2A6, followed by CYP2E1³⁸⁸. NDEA is hydroxylated by CYP2E1 in the liver and other cytochrome P450 enzymes expressed throughout the human body in various tissues and cells. For example, this was demonstrated in scientific

studies as NDEA activation in rats could be prevented by administration of the cytochrome P450 oxidation inhibitors^{358,389}, supporting that NDEA-induced metabolic activation is mediated via cytochrome P450 enzymes.

Studies of NDEA bioactivation and DNA adduct formation have provided important information for understanding species-to-species extrapolation of NDEA and other DNA-reactive rodent carcinogens and their human relevance²⁰². Methylation of DNA adducts such as O6 position of guanine is one of the chemical events that initiates cancer^{390,391}. In studies where NDEA caused cancer in 100% of animals, the highest concentration of the DNA adduct O6-ethyldeoxyguanosine was detectable in the DNA of cells at the earliest time point examined after only 2 days of NDEA treatment³⁹².

Importantly, NDEA can cause cancer at target organs distant to the site of exposure as these bioactivation pathways are present in any of the cells or tissue that express the enzymes that activate NDEA. Humans express cytochrome P450 enzymes (e.g. CYP2E1) similar to rodents and this P450 enzyme is involved in NDEA activation in animals and humans²⁰². Significantly, as in NDMA, the metabolism of NDEA is similar in humans and rodents. CYP2A6 is an isozyme that is important in tumor formation in other species (and may be more prominent) for the bioactivation of NDEA in humans. Based upon the general similarity of NDEA bioactivation and cancer causing activity among species (e.g. including primate monkeys), NDEA will act as a human carcinogen²⁰².

Another enzyme, CYP2A6, is important in human and mice NDEA bioactivation³⁹³. Cytochrome P450s, including CYP2E1, are important in the initial NDEA bioactivation step in the liver by demonstrating inhibition by various agents. One of the important human bioactivating enzymes, CYP2E1, shares 75% of the nucleotides with rat CYP2E1³⁹⁴. After NDEA is bioactivated to an electrophilic ethyldiazonium ion, it undergoes reactions with nucleophiles, including DNA

bases, to form adducts. The bioactivation is effected by several P450 isozymes including CYP2E1²⁰².

It was found that tumor formation in rat liver was proportional to O4-ethyldeoxythymidine formation in DNA, which is proportional to NDEA dose²⁰². Yamazaki et al. found that human liver microsomes can bioactivate NDEA²⁶³. Cytochrome P450 isozymes other than CYP2E1 are responsible for esophageal activation of NDEA⁶³. In fact, the rat esophagus contains CYP2A3 and/or CYP2A6 and an additional unidentified enzyme that can bioactivate NDEA^{395,396}. NDEA is degraded by the action of the cytochrome P450-dependent monooxygenase system to form its active ethyl radicals²⁰². Different cytochrome P450-dependent monooxygenases, including CYP2A and CYP2B groups and CYP2E1, are considered to be key enzymes involved in the activation of NDEA. Aitio et al. (1991) found individual NDEA bioactivation differences within a group of Wistar rats, measured by N-demethylase activity, which correlated with the NDEA-induced tumorigenic response³⁹⁷. The studies have shown that NDEA triggered tumors in every animal species and strain investigated and that NDEA activation can occur in any tissue, organ, or cell which expresses the cytochrome P450³⁹⁸.

As cited above, there is overwhelming evidence that NDEA is metabolically activated via the formation of DNA adducts to cause cancer. The formation of DNA adducts such as O6-ethyldeoxyguanosine as well as O4- and O6-ethyldeoxythymidine, and active ethyl radical metabolites that initiate carcinogenesis is critical to the cancer-causing activity of NDEA. DNA adducts are metabolically activated to electrophiles to exert NDEA's cancer-causing activity. Due to the compelling evidence in the animal assays, animal *in vitro* and human tissue, it is my opinion that NDEA is a human carcinogen via key characteristic #1.

Thus, it is biologically plausible that NDEA causes cancer via key characteristic #1.

Key characteristic #2: NDEA is Genotoxic.

Similar to NDMA, NDEA is genotoxic and mutagenic³⁹⁹⁻⁴⁰². In contrast, few non-carcinogens are mutagenic³⁹⁹. NDEA is shown to trigger a genotoxic response⁴⁰³. The term “genotoxic” refers to a chemical that causes DNA damage, alteration to the genome (mutation), or both⁴⁰⁴. NDEA is genotoxic as it causes DNA damage (e.g., Comets) in all cell types tested including hepatocytes, blood lymphocytes and bone-marrow cells as shown in the Comet assay reflected by increased tail length⁴⁰⁵. The Comet assay is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. DNA fragmentation is a marker for genotoxic effects, and is confirmed by the micronucleus assay and chromosomal aberration counts⁴⁰⁶. NDEA causes genomic damage in exposed cells. As a consequence, the damaged cells may be triggered to proliferate, leading to the formation of cancerous cells that showed increased cell proliferation, angiogenesis, invasion and metastasis. NDEA causes a significant increase in micronucleus induction and DNA fragmentation⁴⁰⁶.

DNA damage induced by NDEA was demonstrated in nasal, oropharyngeal and laryngeal mucosal cells, but also in peripheral lymphocytes⁴⁰⁷. NDEA induces single-strand DNA breaks⁴⁰⁸, which is a clear form of DNA damage. DNA migration in lymphocytes of patients with cancer, as assessed by the Comet assay, was elevated after exposure of lymphocytes to NDEA⁴⁰⁹. These studies show that NDEA can initiate cancer via genotoxic effects.

DNA damage induced by NDEA increases micronuclei due to DNA breakage which could not be repaired, leading to an increase in chromosomal aberrations and apoptotic cell death which can lead to cancer^{186,406}. Compared to the normal tissue DNA, NDEA-induced tumors exhibited significant formation of random DNA fragments⁴¹⁰. Genotoxicity studies with NDEA were

positive for chromosomal aberrations in rat liver cells⁴¹¹ and NDEA-induced DNA single-strand breaks were found in tumor cells (e.g. hepatoma cell lines).

A mutation is a change in the DNA sequence and usually results from the cell attempting to repair the DNA damage²⁴. Gene or point mutations are changes in nucleotide sequence within a gene²⁴. All of these types of DNA damage may give rise to permanent changes in the nucleotide sequence (mutation) as the cell attempts to repair the damage. The DNA adducts (described in key characteristic #1) formed by NDEA (and NDMA), can lead to mutations unless the DNA damage is repaired⁴¹².

Mutagenicity tests are used to indicate potential carcinogenesis risks³⁸³. NDEA is known to be mutagenic at low concentrations³⁸³. NDEA exhibited mutagenic activity with a positive Ames test and a positive SOS chromotest assay^{413,414}. The Ames test is a widely employed biological assay using bacteria to test whether a given chemical causes mutations in the DNA of the test organism. A positive Ames test indicates that NDEA is mutagenic and can act as a carcinogen because cancer is linked to mutations. The Ames test is a quick and convenient test to estimate the carcinogenic potential of a compound as standard carcinogen assays on rodents (e.g., mice and rats) are time-consuming (taking two to three years to complete) and are expensive. The SOS chromotest is an assay that also tests for the genotoxic potential of chemical compounds. This test was developed as a complement or alternative to the traditional Ames test, which involves growing bacteria on agar plates and comparing natural mutation rates to mutation rates of bacteria exposed to potentially mutagenic chemical. Both the Ames assay and the SOS chromotest are standard, useful tools to screen genotoxic and mutagenic chemicals that can cause cancer in humans.

NDEA is mutagenic in the above tests for bacteria *S. typhimurium*, *E. coli*, and *Neurospora crassa*, and produced mitotic recombination in *S. cerevisiae*, recessive lethal mutations in *D. melanogaster*, and chromosomal aberrations in mammalian cells. Positive responses in bacterial cells required a mammalian metabolic system (Montesano and Bartsch, 1976). Yamazaki et al. also obtained a positive genotoxic response by NDEA with *Salmonella typhimurium* NM2009 using human and rat liver microsomal activation systems⁴¹⁵. In the presence of a rat liver microsomal system *in vitro*, NDEA induced chromosomal aberrations and sister chromatid exchanges in Chinese hamster cells⁴¹⁶. NDEA is mutagenic causing forward mutations and reverse mutations³⁸⁷. For these reasons, NDEA is positive in standard genotoxicity and mutagenic tests.

NDEA also causes breaks in chromosomes (“clastogenic”) as severe clastogenicity (> 50% of cells examined showing aberrations) was observed for NDEA⁴¹⁷. Mutations after NDEA exposure were found at high frequency in the K-ras oncogene of lung tumors in mice⁴¹⁸. NDEA is mutagenic as it is a potent inducer of gene mutations and micronuclei. The genotoxic and mutagenic activity of NDEA is neutralized by inhibitors of various cytochrome P450 (including CYP2E1), suggesting that CYP2E1 can mediate the genotoxic and mutagenic activity of NDEA⁴¹⁹. This supports that NDEA is actively genotoxic and mutagenic, except in scientific studies containing cytochrome P450 inhibitors.

DNA fragmentation is a marker for genotoxic effects, confirmed by the micronucleus assay⁴⁰⁶. NDEA increased the number of micronucleus cells⁴⁰⁶ and caused a significant increase in micronucleus induction and DNA fragmentation⁴⁰⁶. That NDEA increases the level of fragmented DNA in the liver of NDEA-treated rats shows the genotoxic activity of NDEA which can lead to initiation of carcinogenesis⁴²⁰. Any agent capable of reacting with DNA to chemically modify it can cause cancer⁴¹³. NDEA also generates 8-hydroxyguanine⁴²¹ (8-OHG), an indicator

of oxidative damage to DNA, which stimulates oxidative stress (key characteristic #5). Accordingly, the key characteristics of carcinogens often synergize with each other to result in NDEA- and NDMA-induced cancers.

The genotoxicity of NDEA is well established. Given the evidence of genotoxicity, it is my opinion that NDEA causes cancer in humans, as well as other species, due to its genotoxic effects via key characteristic #2.

Thus, it is biologically plausible that NDEA causes cancer via key characteristic #2.

Key characteristic #3: NDEA alters DNA repair and causes genomic instability for agents that inhibit DNA repair

Since tumor initiation can be caused by the replication of an unrepaired DNA lesion, DNA repair enzymes have an important role in preventing cancer¹⁰⁷. Several chemical and physical agents have been shown to inhibit DNA repair and/or activate error-prone DNA repair pathways leading to genomic instability and cancer. Carcinogens can act not only by producing DNA damage *directly*, but also by altering the processes that *control* normal DNA replication or repair of DNA damage²⁴. Accordingly, DNA repair activity is a useful human biomarker for mutagenic agents. Genomic instability is a hallmark of cancer and mutations in DNA repair genes provide the basis for the genomic instability⁴²². The biological processes indicating genomic instability include chromosome aberrations, gene mutations, microsatellite instability, and apoptosis²⁴. In addition, genomic instability can be induced by chronic inflammation (key characteristic #6) independently of DNA damage²⁵.

The ability of the tissue to repair DNA adducts play an important role in the mechanism of NDEA causing cancer. Importantly, NDEA alters DNA replications and promote subsequent DNA damage, thereby priming cells for carcinogenesis. NDEA alters DNA repair which can lead to

genomic instability by increasing the micronucleus induction close to the mitotic index, revealing that DNA repair is inactivated⁴⁰⁶.

The DNA damage induced by NDEA corresponds to an increase in micronuclei due to DNA breakage that cannot be repaired, leading to an increase in chromosomal aberrations, and an increase in apoptotic dead cells (key characteristic #10)⁴⁰⁶. When cells (e.g., hepatocytes) were incubated with NDEA, DNA repair was inactive⁴⁰⁶. Studies show NDEA disrupted DNA/replication leading to cancer (e.g. liver)²²⁷ and that NDEA altered (DNA) repair/replication³²⁵. NDEA provoked a significant increase in micronucleus induction and DNA fragmentation which came close to the mitotic index, again revealing that DNA repair is inactivated⁴⁰⁶.

In summary, NDEA alters the processes that control normal DNA replication or repair of DNA damage. Thus, NDEA induces genomic instability with an increased risk for DNA mutations and other genetic changes during cell division. For these reasons, it is my opinion that NDEA is a human carcinogen via alterations in DNA repair capacity and genomic instability.

Thus, it is biologically plausible that NDEA causes cancer via key characteristic #3.

Key characteristic #4: NDEA induces epigenetic alterations (e.g., DNA methylation)

The term “epigenetic” refers to stable changes in gene expression (e.g., DNA methylation) and chromatin organization that are not caused by changes in the DNA. Many carcinogens alter DNA methylation status via epigenetic changes. Epigenetics is the study on how the environment can cause changes in the way our genes work. Unlike genetic changes, epigenetic changes are reversible and do not change your DNA sequence, but they can change how your body reads a DNA sequence. DNA methylation is common epigenetic signaling tool that cells in the body use

to lock genes in an “off” position. DNA methylation is an important process to preserve normal DNA and chromosome functions, so an error in methylation can lead to cancer.

Both NDEA and NDMA induced DNA methylation (e.g. O6-etG) in animals compared to animals treated with a single dose of NDEA or NDMA alone⁴²³. NDEA also induced epigenetic alterations in a study of liver histone profiles in NDEA-induced foci, dysplastic and neoplastic nodules and stages of cancer (e.g., liver) in experimental rats⁴²⁴. A histone is a protein that provides structural support to a chromosome. NDEA caused high expression of the major histone H2A variant H2A.1 during the process of carcinogenesis⁴²⁴. These studies on the role of NDEA in altering specific histone variant support NDEA-induced cancer via epigenetic changes⁴²⁴. Thus, NDEA can act as a human carcinogen via epigenetic alterations.

As NDEA can disrupt epigenetic mechanisms as a carcinogen in humans and animals via DNA methylation. This epigenetic disruption lends further evidence to my opinion that NDEA can cause cancer via key characteristic #4.

Thus, it is biologically plausible that NDEA causes cancer via key characteristic #4.

Key characteristic #5: NDEA induces oxidative stress and subsequent cellular injury

Oxidative stress is a critical tumor promoting mechanism and can lead to oxidative damage to DNA¹⁶⁵. NDEA triggers oxidative stress as even a single dose of NDEA stimulates oxidative stress within 48 hours⁴²⁵. A single dose of NDEA stimulated levels of oxidative stress markers such as hepatic lipid peroxidation (LPO) and conjugated dienes in the rats⁴²⁶. NDEA-stimulated reactive oxygen species (ROS) results in oxidative stress markers in DNA, proteins, and lipids leading to carcinogenicity and mutagenicity^{341,427}.

Oxidative stress results from an imbalance between molecules that stimulate oxidative stress such as reactive oxygen species (ROS) and their elimination by protective mechanisms.

Increased oxidative stress can promote chronic inflammation and cancer. NDEA-derived reactive oxygen species stimulate chronic inflammation (key characteristic #6) via oxidative stress.

Reactive oxygen species, which can arise from inflammation, can contribute to genomic instability and along with other free radical species play key roles in many of the processes identified as being necessary for the conversion of normal cells to cancer cells^{13,23}. NDEA stimulates the release of pro-inflammatory molecules (e.g. cytokines and nitric oxide) from blood cells activated lead to oxidative stress via pro-inflammatory pathways⁴²⁸. NDEA causes oxidative stress via reactive oxygen species (ROS) via cytochrome P450s (e.g., CYP2E1)⁴²⁹. These reactive oxygen species (ROS) causing oxidative stress damage by NDEA is toxic to the cells leading to tumor promotion^{426,429}.

Importantly, NDEA also reduces anti-oxidative stress markers (antioxidants) such as glutathione reductase (GSH-R) activity as well as total glutathione (GSH) content in the rat liver⁴²⁶. The first line of defense to protect from oxidative stress is to block reactive oxygen species via antioxidants that prevent or reduce oxidative stress. Cells have evolved a series of antioxidant systems to handle these dangerous natural by-products including superoxide dismutases (SODs). Oxidative damage to molecules such as DNA, proteins, and lipids arising from redox imbalances, is normally defended against by enzymes that block oxidative stress (SOD, CAT, GSH-Px, GSH-Red, and Glc 6-PD)⁴²⁰.

The reactive metabolites of NDEA and the free radicals generated by cytochrome P450-dependent enzymes produce oxidative stress, which can lead to cancer³⁴⁵. Lipid peroxidation can result in the formation of several byproducts of oxidative stress, such as malondialdehyde (MDA) and 4-hydroxynonenal. These oxidative stress molecules can attack cells including DNA, thereby

promoting mutagenicity and carcinogenicity⁴³⁰. NDEA causes a reduction in ROS detoxifying enzymes (SOD, CAT, GSH-Px, GSH-Red, and Glc 6-PD) resulting in excessive generation of ROS^{348,420}. Thus, these NDEA-induced reductions could lead to oxidative stress resulting in tumor promotion.

NDEA stimulates oxidative stress in lipids via an increase in the levels of lipid peroxidation products (conjugated dienes, lipid hydroperoxides, and malondialdehydes)⁴²⁰. This key characteristic of carcinogens is not specific to cancer and occur in other inflammatory diseases such as stroke, Alzheimer's disease, Parkinson's disease, and diabetes. Thus, oxidative stress is best analyzed in the context of its impact on other key characteristics such as genotoxicity (key characteristic #2) and chronic inflammation (key characteristic #6). NDEA causes oxidative stress and cellular injury via free radicals^{383,431}. NDEA is so potent in generating oxidative stress including reactive oxygen species it is used as a gold-standard to evaluate detoxification and ROS scavenger drugs or reagents that may prevent oxidative stress such as medicinal plants⁴²⁰.

NDEA causes oxidative stress, oxidative DNA damage and cellular injury by generating reactive oxygen species (ROS) leading to cancer progression^{341,345,425}. NDEA stimulates the levels of oxidative stress markers like lipid peroxidation (LPO), protein carbonyl (PCC), and glutathione-S-transferase (GST) activity and reduces total glutathione (GSH) content⁴³². NDEA stimulates oxidative stress markers including malondialdehyde, conjugated dienes, lipid hydroperoxides, protein carbonyl, and percentage DNA fragmentation⁴²⁰. Exposure to NDEA resulted in a significant decrease in the level of the antioxidants glutathione such as GSH in the liver of rats when compared to the control⁴²⁰.

NDEA impaired antioxidative defense is reflected by a significant elevation in the level of oxidative stress marker (MDA) and a significant depletion of free radical scavenging antioxidants

(GR, GPx, SOD and GSH)⁴³³. NDEA lowers the antioxidant levels in both liver and serum in animals³⁴¹. NDEA also lowers the antioxidant glutathione (GSH)³⁴⁰. NDEA causes the generation of ROS resulting in oxidative stress and cellular injury¹²⁷.

To maintain cellular health in the human body it is essential to have a reactive oxygen species (ROS) scavenger system that blocks oxidative stress³⁴⁵. Antioxidants have been tested in the NDEA-induced oxidative stress models to study the anti-oxidative protective effects of agents such as Vitamin E, ellagic acid, quercetin, curcumin, apigenin, turmeric and garlic powder. These agents all counteract NDEA-induced oxidative damage by reducing or preventing oxidative stress^{350,351,426,427,434}. NDEA-induced hypoxia also triggers oxidative stress via the production of ROS which further activate HIF-1alpha⁴³⁵. NDEA-induced cancer formation was associated with alterations in the hypoxia related proteins such as HIF-1a and HOX. A significant rise in HIF-1a protein expression followed by a concomitant decrease in HOX in NDEA-treated group increases hypoxia and subsequent tumor angiogenesis (key characteristic #10) and metastasis⁴³⁵. The imbalance between antioxidant defense system and generation of reactive oxygen species leads to oxidative stress. Therefore, investigations were made on the antioxidant status of the experimental animals exposed to NDEA⁴³⁶. Superoxide dismutase (SOD) is an enzyme that incurs prominent defensive mechanism against oxidative stress and it was found that NDEA significantly declines the oxidative stress protection factor (SOD) within 14 days of its treatment.

This decline in NDEA-induced activity leads to the generation of superoxide radicals and further oxidative stress⁴³⁷. NDEA induced oxidative stress by lipid peroxidation (LPO) and protein carbonyl formation³⁶⁵. Oxidative stress-induced DNA damage has been measured by single cell gel electrophoresis (Comet assay)³⁶⁵. The NDEA-induced oxidation of DNA in human cells occurs as a consequence of attack by free radicals when oxygen radicals attack DNA bases and

deoxyribose residues, producing damaged bases and single strand breaks increasing tumor promotion.

As a result, NDEA can promote or cause cancer by stimulating oxidative stress via oxygen radical-induced cellular injury which provides evidence to support my opinion that NDEA can cause cancer in humans via oxidative stress and key characteristic #5.

Thus, it is biologically plausible that NDEA is a human carcinogen via oxidative stress in key characteristic #5.

Key characteristic #6: NDEA induces chronic inflammation

Many experimental studies including from my laboratory have confirmed that inflammation can stimulate or induce tumor initiation, growth, and metastasis^{6-10,13,33,182-186}. Inflammation in the tumor microenvironment is now known as a hallmark of cancer¹⁰¹ and is recognized as a key characteristic of carcinogens^{24,25}.

Chronic inflammation promotes tumor growth and progression⁴³⁸. NDEA increased pro-inflammatory cytokines such as TNF-alpha which can promote tumor growth⁴¹⁰. My laboratory and others have shown that chronic inflammation can initiate and promote various cancers^{11,340}.

NDEA increases tumor markers alpha-feto protein (AFP) via stimulation of the pro-inflammatory cytokines TNF-alpha and IL-6 in the blood³²³. NDEA-stimulated cytokines (i.e., TNF-a and IL-6) play a critical role in stimulating tumor cell proliferation and angiogenesis (key characteristics #10)³²³. NDEA-increased pro-inflammatory cytokines reflect an aggressive inflammatory response which promotes tumor growth. These pro-inflammatory molecules such as cytokines act as growth factors to stimulate proliferation, inflammation, angiogenesis, and transformation of tumor cells. Unresolved inflammation is a driving force behind the development

and acceleration of various cancer types including liver, prostate, pancreatic, lung, blood cancers (e.g., lymphoma and leukemia), colon, and head and neck (e.g., esophageal)^{439,440}.

NDEA stimulates inflammation as determined by histopathologic analysis showing mononuclear inflammatory cells³²³. NDEA stimulates pro-inflammatory cytokines IL-1B, IL-2, IL-6, and IL-10 in the tumor tissue³⁴⁰. In fact, NDEA significantly stimulates by twofold to threefold these pro-inflammatory cytokines in rat hepatic tumor tissue. IL-6 is a key procancer cytokine that stimulates inflammation-associated cancers to promote tumor progression, invasion and metastasis⁴⁴¹.

Histological examination of liver tissue done under light microscope can observe the effect of NDEA promoting inflammation on the cells. For example, the liver tissue from NDEA-treated animals showed increased acute inflammatory cells compared to normal histological appearance of liver cells in animals not exposed to NDEA⁴⁴². On histopathological analysis, NDEA-treated rats exhibit an excess inflammation (e.g., inflammatory cells called neutrophils), necrosis, and excessive collagen in the tissues⁴³⁶. Thus, inflammation such as in the liver is prominent in the NDEA-treated group.⁴³⁶ Time-dependent severity of liver inflammation followed by fibrosis is associated with NDEA-increased liver enzymes (e.g., AST, ALT, ALP, γ GT and bilirubin levels). NDEA upregulates the pro-tumorigenic and pro-inflammatory molecule called NF-kB which results in transcription of genes that contribute to cancer via non-genotoxic mechanisms such as cell proliferation, inflammation, oxidative stress and angiogenesis. NDEA increased expression of NF-kB can stimulate tumor growth by promoting inflammation.

NDEA functions as a tumor promotor by stimulating chronic inflammation. By stimulating inflammation, NDEA can accelerate tumor growth by stimulating other key carcinogen characteristics such as oxidative stress, immunosuppression, angiogenesis, and apoptosis (cell

death)⁴³⁹. Importantly, chronic inflammation can promote genotoxicity, oxidative stress and genomic instability. Because NDEA stimulates all of these key characteristics, inflammation can act synergistically with these other key characteristics to promote many types of cancer.

This evidence adds support to my opinion that NDEA can cause and promote human cancer as a carcinogen.

Thus, it is biologically plausible that NDEA is a human carcinogen via chronic inflammation under key characteristic #6.

Key characteristic #7: NDEA is immunosuppressive

Immunosuppression is important to the mechanism of carcinogenesis since the immune system of the human body normally functions to protect against cancers. Chemicals that cause immunosuppression can cause or stimulate cancer. Carcinogens can cause or promote by suppressing the immune system. For example, NDEA induced severe anemia in the hen's egg⁴⁴³ which results in decreased healthy red blood cells to carry adequate oxygen to the body's tissues. Anemia can adversely affect the immune system. If a carcinogen such as NDEA enters the bone marrow it can induce immunosuppression.

Chronic inflammation also induces immunosuppression via induction of proinflammatory mediators and accumulation/activation of immune suppressor cells¹³. Immunosuppression can result from a pro-inflammatory immune response. NDEA strongly affected cellular immune response pathways²⁸⁴ and when tested on gene expression transcriptomic profiling, it showed increased pathways of pro-inflammatory immune response which can promote cancer²⁸⁴. NDEA influenced pathways involved in the stimulation of proinflammatory cytokines, including the IL-1 and IL-6 signaling pathways²⁸⁴. Cytokine production, especially IL-6, plays an important role in

the induction of the acute phase response and stimulation of the intestinal inflammatory response⁴⁴⁴. NDEA has also been shown to cause poorly differentiated carcinomas in immunosuppressed new-born rats⁴⁴⁵.

Since NDEA can disrupt the normal host immune response, which protects from cancer progression, via tumor-promoting inflammation. The ability of NDEA to be immunosuppressive contributes to the pro-carcinogenic activity of NDEA. NDEA notably causes dysfunction of the immune system, which plays a critical role in carcinogenesis.

Thus, it is biologically plausible that NDEA causes cancer via immunosuppression under key characteristic #7.

Key characteristic #8: Modulates Receptor-Mediated Effects

The carcinogen NDEA receptor-mediated activity has not been characterized. Receptor-mediated effects can occur at the cell surface (through ligand-binding) or intracellularly (via the disruption of signaling cascades or actions on nuclear/cytosolic receptors), all of which can modulate transcriptional changes in the nucleus. Thus, both receptor binding and receptor functional activity of NDEA has not been characterized and it is unknown if NDEA exhibits this characteristic.

Key characteristic #9: Causes immortalization

Cancer cells are immortal, and therefore have limitless replicative potential. In contrast, normal cells have a limited lifespan. Immortalization is associated with stemness, the ability of cells to self-replicate indefinitely and to transform into various cell types. The opposite of immortalization and stemness is cellular senescence, a cellular program in which cells stop

dividing. Chemical carcinogens including tobacco, PCBs and asbestos promote immortalization and inhibit senescence.

Morphological transformation of hamster, mouse, or rat non-cancer cells in models of chemical carcinogenesis are a reliable index of tumorigenic potential or the ability of cells to form progressively growing tumors when injected into an animal^{446,447}.

Transformation does not usually occur when exposing non-cancer cells to noncarcinogens⁴⁴⁷. NDEA causes induced malignant transformation of human epithelial cells to result in transformed cells with a prolonged life span⁴⁴⁵. NDEA was shown to form anchorage independent colonies in soft agar⁴⁴⁵, which is a standard assay to test the transformation of normal cells into cancer cells. The soft agar colony formation assay is a well-established method for characterizing the ability of normal cell in culture (*in vitro*) to change into cancer cells and is considered to be one of the best tests for the ability of a normal cell to transform to a cancer cell⁴⁴⁷. In these studies, NDEA-transformed cells exhibit prolonged life span, aneuploid karyotypes, and form anchorage-independent colonies in soft agar, which are qualities of cancer cells⁴⁴⁵.

NDEA also causes the malignant transformation (altered morphology, extended life span, anchorage independent growth, invasiveness, and tumorigenicity, etc. of normal cells called fibroblasts derived from *human* fetal lung⁴⁴⁸. For this reason, NDEA is an effective carcinogen used in research to induce the malignant transformation of human cells such as fibroblasts into cancer cells⁴⁴⁸ and is utilized to study the mechanisms of carcinogenesis²⁰².

NDEA exposure also caused the transformation of anchorage-independent cells by DNA synthesis in a fetal *human* tracheal epithelial cell line⁴⁴⁹. NDEA stimulated the frequency of anchorage-independent colonies grown in soft agar which was directly related to the dose of NDEA⁴⁴⁹. Colony-forming efficiency, as an expression of the cell transformation effect, was also

dependent on the dose of NDEA⁴⁴⁹. NDEA was found to transform normal guinea pig fetal cells in culture⁴⁴⁷. NDEA increased also oxidative stress levels (key characteristic #5) of SOD and CAT enzymes contribute to cancer transformation from normal cells³⁴⁰.

A high profile publication of a study in the high impact journal Nature created a bioassay system that was modified by intraperitoneal injection of NDEA into pregnant hamsters on day 12 of gestation followed by removal of the embryos on day 14, a period that allowed for completion of metabolic interaction⁴⁴⁶. These transformed cells were produced at a much higher frequency than in control cultures from untreated embryos, and they produced tumors (fibrosarcomas) when injected back into animals⁴⁴⁶.

The immortalization evidence of the effects by NDEA on cells adds evidence to support my opinion that NDEA can act as a human carcinogen by stimulating immortalization.

It is biologically plausible that NDEA causes cancer via immortalization under key characteristic #9.

Key characteristic #10: NDEA alters cell proliferation, cell death, or nutrient supply (e.g., angiogenesis)

Sustained cellular proliferation is a key factor in cancer progression. As summarized in the United States Environmental Protection Agency guidance assessing risk of cancer from early-life exposures (EPA, 2005), more frequent cell division during development can result in enhanced fixation of mutations because of the reduced time available for repair of DNA lesions, while clonal expansion of a mutated cell produces a larger population of mutant cells²⁵. Cell death releases pro-inflammatory signals into the surrounding tissue microenvironment, resulting in recruitment of inflammatory cells of the immune system that can participate in tumor promotion through their influence on cancer cell proliferation and invasiveness. Angiogenesis, in which new blood vessels

grow into a tumor, is key to providing nutrients to the cancer. Tumor growth requires angiogenesis to grow¹.

NDEA can alter cell proliferation via cell toxicity activity. NDEA induced *uncontrolled* proliferation in the liver of NDEA treated mice which was evident from the high expression of cell-proliferation associated genes (PCNA, Cyclin D1, and p21) when compared to control³⁵⁷. In another study, NDEA stimulated a higher rate of cell proliferation, which was further evident from increased *PCNA* and *Cyclin D1* expression³⁵⁷. NDEA has induced reduced expression of p21, which blocks proliferation to prevent tumor progression³⁵⁷.

Exposures of NDEA-initiated cells to chemicals with tumor-promoting activity can enhance the development of such tumors by enhancing the proliferation of the initiated cells. At higher doses, the cytotoxicity of NDEA can stimulate cell proliferation, which can increase tumorigenicity. The mutagenic effects of NDEA-induced DNA adducts can also stimulate cell proliferation at rates greater than the cells of the surrounding tissues, thereby establishing a collection of altered cells, which can develop into tumors.

Enhanced cell proliferation by carcinogens increases the rate of the development of initiated cells²⁰². Increased cell proliferation, which is measured by increased DNA replication, has been found for normal cells and for altered hepatic foci during chronic NDEA administration²⁰². In Peto et al, NDEA-induced cell proliferation was important to tumorigenesis in the esophagus¹⁹. In rat liver and esophagus, NDEA increased the tumor proliferation rate resulting in hyperplasia and cancer. Altered foci, induced by NDEA, exhibited about a 10-fold increase in the rate of cell proliferation. Within a few days of NDEA exposure, single hepatocytes that were GST-I(+) had a much greater probability of enhanced DNA synthesis than unaltered hepatocytes. After this NDEA-induced DNA damage, the number of phenotypically altered and

labeled cells increased dramatically in relation to unaltered hepatocyte, providing evidence of cell proliferation.

An interval of NDEA-induced cell toxicity was found by Rajewski who exposed male rats to NDEA. NDEA initially reduced liver cell proliferation to 2/3 control rate for 3-5 days. After this period, cell proliferation increased to 2-3 times the control value. This initial depression of DNA synthesis before an eventual cell proliferative increase resulted from NDEA-induced cell damage or death that accumulated before a regenerative response was triggered^{218,450}.

Increased cell proliferation with increasing NDEA dose rate was found by Rajewsky (1972), who measured the fraction of DNA synthesizing rat liver cells as a function of NDEA dose rate continuously orally administered to male BDIX rats⁴⁵¹. A maximum cell proliferation for NDEA exposed rats was 85%.

Dose-response for NDEA-induced liver-cell proliferation was studied by Deal et al. (1989) using 6-week-old male F344 rats exposed to NDEA in drinking water for 1, 4 or 10 weeks. Exposure to NDEA increased cell proliferation by 300% and 400% in all lobes. The minimum dose required for enhanced cell proliferation correlated with the 1 ppm dose in male rat liver, where there was an increase in dose-response at high-dose exposure compared with low-dose exposure⁴⁵². NDEA increases hepatocyte proliferation^{202,323}. Thus, NDEA-initiated cells have much higher cell proliferation rates than the surrounding hepatocytes.

Tumor initiation, progression, and maintenance commonly involve alterations in cell death (e.g., apoptosis). Studies have shown that dysregulation of cell death is an important cause for the cancer formation. Two forms of cell death include apoptosis and necrosis. NDEA stimulates necrotic tissue damage with cell death³⁴⁰. A single dose exposure of NDEA causes massive activation of proapoptotic mechanisms⁴⁵³. NDEA induces cell death including apoptosis and

necrosis⁴⁰⁶. Metabolites generated from the metabolic activation of NDEA can induce DNA damage that is not repaired, thereby resulting in cytotoxicity and subsequent cell death⁴⁰¹. NDEA also stimulated necrotic cell death in liver cancer in mice⁴¹⁰. These necrotic dead cells also recruit inflammatory cells of the immune system to their place.

The immune inflammatory cells can act as tumor promoters since these cells can help in cancer cell proliferations and foster angiogenesis⁴¹⁰. Angiogenesis is the growth of new blood vessels and is important tumor promotion as tumors require nutrients from the blood supply. Many tumors including liver, lung, bladder, colon, prostate, pancreatic, blood, and esophageal, are angiogenesis-dependent¹. Significant elevation in angiogenesis associated with vascular endothelial growth factor (VEGF) and CD31 proteins were observed in NDEA-treated mice compared to the mice that were not exposed to NDEA control group⁴³⁵. VEGF is a potent angiogenesis factor that can stimulate many types of cancer including liver, prostate, bladder, lung, colon, blood, pancreatic, and esophageal cancers¹. CD31 is a marker of angiogenesis cells called endothelial cells in the tumor⁴. NDEA stimulates tumor angiogenesis in the mice as determined by the increased levels of the angiogenesis markers (i.e., VEGF and CD31 at 10 weeks of NDEA treatment)⁴³⁵. NDEA treatment in the mice significantly raised the pro-angiogenic molecules called matrix metalloproteinases (MMPs) including MMP-9 and MMP-2 in comparison with the control⁴³⁵. For a cancer cell to spread or metastasize from the original primary tumor to other organs, it must locally degrade the tissues. The key molecules allowing the breakdown or degradation of the tissue to allow the cancers to spread are called matrix metalloproteinases (MMPs)⁴⁵⁴. Thus, NDEA can act as a tumor promotor by altering these 3 critical processes: cell proliferation, cell death and the vascular supply (angiogenesis).

It is biologically plausible that NDEA acts as a tumor promotor via cell proliferation, cell death and the angiogenesis (vascular supply of nutrients) under key characteristic #10.

Therefore, similar to NDMA, NDEA is a potent carcinogen which exhibits 9 of the 10 key characteristics of carcinogens. Given the compelling scientific evidence of carcinogenic activity in animals, evidence in human cell and tissue, substantial evidence of genotoxicity and knowledge of the many biologically plausible mechanisms of carcinogenicity of NDEA, **NDEA is a human carcinogen.**

NDMA and NDEA are similar nitrosamines

Among the *N*-nitrosodialkylamines, NDMA and NDEA are the structurally simplest and the most prevalent ones⁴⁵⁵. Importantly, both are alkylating that introduce alkyl radicals into biologically active molecules and thereby prevent their proper functioning. Similar to NDMA, NDEA exhibits a *no-threshold* implying that a carcinogenic response for NDEA can occur at any dose¹⁹. The National Library of Medicine, National Center for biotechnology Information describes N-Nitrosodiethylamine (NDEA) as is a synthetic light-sensitive, volatile, clear yellow oil that is soluble in water, lipids, and other organic solvents, and explains that N-Nitrosodiethylamine affects DNA integrity, probably by alkylation, and is used in experimental research to induce liver tumorigenesis. **It is considered to be reasonably anticipated to be a human carcinogen; a nitrosamine derivative with alkylating, carcinogenic, and mutagenic properties**²⁰² (National Library of Medicine, National Center for Biotechnology Information, N-Nitrosodiethylamine. <https://pubchem.ncbi.nlm.nih.gov/compound/5921>).

When this description is compared to that of NDMA, the similarities are readily apparent: N-Nitrosodimethylamine (NDMA) is described a volatile, combustible, yellow, oily liquid nitrosamine with a faint characteristic odor that decomposes when exposed to light and emits toxic

fumes of nitrogen oxides when heated to decomposition. N-Nitrosodimethylamine is primarily used in laboratory research to induce tumors in experimental animal. **This substance is reasonably anticipated to be a human carcinogen. A nitrosamine derivative with alkylating, carcinogenic, and mutagenic properties** (National Library of Medicine, National Center for Biotechnology Information, N-Nitrosodimethylamine. <https://pubchem.ncbi.nlm.nih.gov/compound/6124>).

These two nitrosamines have been extensively investigated for their potent cancer causing mechanisms of action⁴⁵⁵. NDMA and NDEA are both metabolized by cytochrome P-450 enzymes which are expressed by many tissues, organs, and cells throughout the body⁴⁵⁶. The structural difference between NDMA and NDEA is relatively small, especially for the P-450 family of isozymes⁴⁵⁶. Thus, many of the mechanisms of cancer causation detailed for NDMA apply to NDEA.

Explant cultures from a variety of different *human* tissues including bronchus, esophagus, urinary bladder, colon, and pancreatic duct have been used to study nitrosamine metabolism and DNA binding¹⁰⁷. The cultures confirmed that NDMA and NDEA are metabolized similarly in all tissues examined which is supported by observation of DNA methylation in human explant cultures²⁰².

NDEA Oncogene Activation

Similar to NDMA, NDEA-induced cancer can result from the activation of oncogenes and inactivation of tumor suppressor genes (TSG). In mice given a single injection of NDEA at 12-15 days of age, 36% of adenomas and 54% of carcinomas were found to have activated ras genes compared with 0% in normal liver⁴⁵⁷. Activation of proto-oncogenes c-myc and c-jun occurs at

early and late stages of NDEA-induced cancer⁴⁵⁸. These findings explain yet another mechanism for NDEA-induced cancer.

NDEA dose-response demonstrated in animal studies

There are many animal studies that show both NDEA causes cancer via a classic-dose response. Low doses of genotoxic carcinogens including NDEA caused a dose-dependent incidence of liver cancer²²⁵. The studies' results indicate dose dependency of liver tumor formation even at very low exposure levels of NDEA²²⁵.

Peto et al (1991) demonstrating a classic dose-response between exposure to NDEA and the induction of tumors including esophagus in the rat^{19,74}. In the large dose-response study in Colworth rats, tumor incidence data were adjusted to account for the presence of two fatal tumor types, which occurred at high incidences¹⁹. At low doses, Peto et al reported the incidence of liver tumors was linear related to dose⁷⁴. The increased tumor-formation rate at doses > 1 ppm in the large bioassay¹⁹ corresponded to the increased NDEA-induced cell proliferation above 1 ppm in male rat liver⁴⁵². Above 1 ppm NDEA in drinking water, cell proliferation increased in a time- and dose-related manner, which correlated with the increased tumor incidence seen above 1 ppm in male rat liver¹⁹. Consequently, tumor incidence in the liver was related to both adduct formation and cell proliferation rates. The dose-responses for liver and esophageal tumors showed that incidence was exponentially related to dose where tumors were found. Lifetime exposure of BDII rats to NDEA in drinking water at doses increasing from 0.7 to 132.5 ppm produced dose-related decreases in time to tumor (tumor latency) for both liver and esophagus tumors^{202,315}.

In studies by Clapp et al (1970), NDEA induced tumors in mice including lung adenomas, hepatomas, and forestomach squamous cell carcinomas and maximum incidences reached 84%, 98%, and 100%, respectively, as compared with control values of 41%, 5%, and 0%²¹⁷. At lower

doses, incidence of liver tumors increased linearly with dose, reaching 80–90% at higher NDEA doses²¹⁷. Only at the lowest dose did NDEA not induce a maximum incidence of stomach tumors. The mean survival time and mean age at NDEA-induced death with all types of tumors, and minimum induction times decreased with increasing dose consistent with a dose-response²¹⁷.

In a high profile publication in the prestigious journal called *Science*, NDEA was reported to induce a dose-response of liver tumors (hepatocellular carcinoma) in rats: 92% of rats died from the liver tumors²¹⁸. The NDEA-induced dose response that a single dose of NDEA was irreversible and cumulative²¹⁸. NDEA in the drinking water also induced a dose-response for liver tumors in guinea pigs²¹⁹. No tumors appeared in the groups which were exposed to NDEA for 4-8 weeks while there was a 21% incidence tumor incidence in guinea pigs which were exposed to NDEA for 12 weeks²¹⁹.

In Lijinsky et al rats were exposed to NDEA in drinking water at various concentrations and exposure durations²²². Animals were allowed to die with tumors, and time to death was used as one measure of NDEA potency. Time to death was inversely related to dose so animals exposed to a higher dose died more quickly²²². Dose-related tumors were found in the liver and upper gastrointestinal tract, including the esophagus, forestomach, tongue and oropharynx²²². Similarly, NDEA shows the steepest slope when analyzing the relationship between daily carcinogen doses and time to liver tumor occurrence⁴⁵⁹. NDEA also induced a dose-response in Syrian Golden hamsters in upper respiratory tract tumors such as nasal cavities, larynx, and trachea²²⁰. Nasal cavity tumors developed early and reached incidences varying from 17 to 75%. These tumors included squamous cell papillomas, squamous, adenocarcinoma, anaplastic carcinomas, and neuroepithelial tumors. Tumors of the larynx reached incidences of 17 to 72%, and tumors of the

trachea incidences ranged from 88 to 100%. NDEA also induced dose dependent liver cancer in rainbow trout²²³.

The principal relationships studied have been effects of NDEA dose on time to death, time to tumor or tumor incidence. In one study a dose-related increase in tumor incidence with an apparent no-observed-effect-level (NOEL) at 0.26 ppm was found for NDEA-induced esophageal tumors¹⁹. Female dose-response occurred for all liver and esophageal tumors, both fatal and incidental, malignant and benign. There was a large number of both liver and esophageal tumors. The incidence of liver neoplasms was reported to be directly proportional to dose¹⁹. The authors concluded that among rats allowed to live their natural life span, doses of 0.01, 0.1 and 1 ppm NDEA caused about 0.25, 2.5 and 25% of exposed rats to develop liver neoplasms above the control rate, with no indication of any threshold¹⁹.

Monkey tumorigenesis related to NDEA exposure was examined by Thorgeirsson et al. (1994)²²⁴. NDEA was the most potent and predictable hepatocarcinogen in cynomolgus, rhesus, and African green monkeys. However, when administered intraperitoneally to galagos (a prosimian), NDEA induced primarily mucoepidermoid carcinoma of the nasal cavity²²⁴. Thus, *NDEA was judged to be the most predictable and potent hepatocarcinogen of all the agents tested*, including 2-acetyl- aminofluorene, aflatoxin B, and NDMA²²⁴. In a 10-year study, NDEA was given intraperitoneally to newborn rhesus, cynomolgus, African green and rhesus-cynomolgus hybrid monkeys at varying doses every 14 days until a tumor was detected. There was a linear relationship between dose and tumor latency²²⁴.

Thorgeirsson et al. (1994) also administered NDEA intraperitoneally to 14 prosimian bushbabies²²⁴. After 10 years, 10 of the 14 have developed mucoepidermoid carcinoma of the nasal cavity and 2 developed liver carcinomas. The average latency period was ~ 22 months²²⁴.

Thus, NDEA studies have made important contributions to the dose-response component of risk assessment for DNA-reactive chemicals in the cancer biology and toxicology fields²⁰². Cells initiated by NDEA have been found to be persistent and do not require the continued presence of NDEA in order to develop into tumors²⁰².

NDEA causes different tumor types in multiple animal species

There is now convincing evidence that the biological activity of nitrosamines including NDEA and NDMA in humans do not differ substantially from that in experimental animals¹⁰⁷. We can therefore predict with high confidence that N-nitroso compounds including nitrosamines such as NDEA and NDMA are carcinogenic¹⁰⁷. We can look to the cancer animal studies to ascertain the types of cancers that can be caused by NDEA.

NDEA is highly carcinogenic as proven by causing cancer in all ten animal species tested, including sub-human primates. The main target organs of NDEA include but are not limited to liver, lung, esophagus, trachea, and nasal cavity. NDEA administered to pregnant mice, rats, and hamsters has even been shown to act transplacentally, inducing tumors in the progeny (Tomatis, 1973; Mohr, 1966)²⁰².

Below are examples of the numerous studies showing NDEA causes a vast variety of cancers.

Liver Cancer

In a *Salmonella typhimurium* cell line, Yamazaki et al. found that both CYP2A6 and CYP2E1, which were isolated from *human* liver microsomes and added to a reconstituted system, were effective in activating NDEA⁴¹⁵. In an ancillary BIBRA study, Gray et al. (1991) examined effects of the same 16 NDEA doses used in the main bioassay on small groups of female mice and hamsters³⁸⁰. In mice, sites with statistically significant numbers of neoplasms were liver,

esophagus and stomach. In hamsters, statistically significant numbers of neoplasms were seen only in liver and trachea. In mouse liver, there was evidence of NDEA-induced hepatocellular and Kupffer cell tumors³⁸⁰.

In hamsters, there were statistically significant liver-cell and bile-duct neoplasms. Three-week-old rats were more susceptible than 20-week-old rats to liver tumors, which was attributed to greater cell proliferation in the weanlings³⁸⁰. NDEA induced multiple squamous-cell papillomas of the trachea and bronchi in hamsters as well as carcinoma of the nasal cavity, liver (e.g. hepatocellular carcinoma) and kidney⁴⁶⁰.

NDEA administered by gavage (force fed), in drinking water, or by feeding produces liver tumors in the following species: rats, mice, hamsters, guinea pigs, rabbits, dogs, and monkeys (Yamamoto et al., 1972; Druckrey et al., 1967, 1963; Magee et al., 1976; Rajewsky et al., 1966; Tomatis, 1973)²⁰². Multiple animal studies have demonstrated that NDEA causes liver cancers on different strains and species. In the liver, mainly haemangioendotheliomas⁴⁶¹ and adenomas²⁵³, but also hepatomas²⁵⁶ were induced. Hepatic (liver) tumors were also generated by intraperitoneal injection of NDMA to male BALB/c mice⁴¹⁰.

In Peto *et al.*, in a very large bioassay at British Industrial Biological Research Association (BIBRA), 4080 inbred Colworth Wistar rats were treated with different concentrations of NDEA¹⁹. Animals were killed only when they were dying or had palpable liver tumors. Peto et al was considered a very robust study, testing 15 NDEA concentrations with a total of more than 2000 animals¹⁹. Peto et al. (1984) exposed groups of 48 Colworth rats/sex to NDEA in drinking water at 15 concentrations between 0.033 and 16.896 ppm. Six animals/group were sacrificed at 6 and at 12 months and the remainder kept on treatment until natural death. Data on tumor incidence was not reported for each group, but data pooled by sex indicated positive trends for tumors of the

nasopharynx, lower jaw, stomach, kidney, ovaries, seminal vesicles, liver, and esophagus. Dose-related increases in incidence of upper GI tumors and liver cell tumors were observed in C57-BO mice, and tracheal and liver cell tumors were observed in Syrian hamsters (Peto et al., 1984).

NDEA induces liver cancer in chickens⁴⁶². In rats, following the first report of liver carcinogenesis in 1960 by oral administered NDEA in rats, many laboratories confirmed the cancer-causing activity of NDEA. In most cases, hepatocellular tumors have been observed, often with lung metastases; in most cases, cholangiomas (related bile duct tumors) have been described^{300,463}. In lifetime feeding studies, tumor yields approach 100%. Liver tumors, including 13 hepatocellular carcinomas, 9 hemangiosarcomas, and a blastoma were caused in 23/25 in 16-week-old male rats by daily administration of 1 mg NDEA in the drinking water⁴⁶⁴.

In guinea pigs, NDEA administered in the drinking water induced hepatocellular carcinomas and liver adenocarcinomas, some metastasizing into the lungs in all of 11 treated animals and 7 of 8 had animals developed liver tumors⁴⁶⁵. In another study, hepatocellular carcinoma was the main tumor-type induced in 14 of the 15 animals (guinea pigs) that lived more than 16 weeks²²¹. Studies of rabbits given daily doses of NDEA in the drinking water induced liver-cell carcinomas in 2 of 2 treated rabbits⁴⁶⁶. In another study, all 13 rabbits exposed to NDEA in the drinking water died with metastasizing hepatic carcinomas; one animal had a carcinoma of the lung⁴⁶⁷.

A study involving dogs found primary hepatic neoplasms of various types were induced in adult male mongrel dogs given NDEA in the drinking water for 2-50 weeks; these consisted of 3 fibromas, 4 leiomyomas, 1 hemangioma, 10 hemangiomaendothelioma, 4 fibrosarcoma, 2 leiomyosarcoma, 1 hepatocellular carcinoma, 1 cholangiocarcinoma and 1 undifferentiated-cell

carcinoma. Six dogs developed squamous cell carcinoma of the nasal cavity⁴⁶⁸. Oral administration of NDEA (0.02% in water) induced liver tumors in 100% of animals³⁴⁴.

Various tumors of the liver, 1 adenoma of the kidney, and 1 squamous-cell carcinoma of the ethmoid, were induced by daily oral doses of NDEA to 4 pigs⁴⁶⁹. In a group of mini-pigs, all 5 mini-pigs who received oral NDEA in aqueous solution developed hepatocellular adenoma. Four pigs developed hepatocellular carcinomas; one of these animals also had a Kupffer-cell sarcoma and another had metastases of the hepatocellular carcinoma found in the lung.

In monkeys, hepatocellular carcinomas were induced in 6/15 rhesus and cebus monkeys treated with various oral doses of NDEA for a year with an induction time between 14 and 24 months⁵⁰. Two (green) monkeys treated intraperitoneally with NDEA every 2 weeks for 26 months developed hepatic-cell carcinomas⁵⁰.



Figure 4. Figure 6. Cynomolgus monkey #177. Liver is cirrhotic and contains numerous soft, white tumor nodules. Monkey had received N-nitrosodiethylamine in his food for 14 months. Figure 12; Rhesus monkey #131. Received N-nitrosodiethylamine orally for 2 years. Liver is greatly enlarged and contains numerous partly confluent, firm, white tumor nodules. Apart from tumor nodules, liver had a finely nodular appearance.



Mucoepidermoid carcinomas in the nasal cavities developed in 10/14 prosimian primates treated by every 2 weeks with NDEA; 2 of 10 animals also had primary carcinoma of the liver²²⁴.

Subcutaneous administration of NDEA to Algerian hedgehogs caused benign and malignant tumors of the liver and lung⁴⁷⁰. In a bird study, grass parakeets were injected once a week and 6/9 birds that survived the treatment died with malignant hepatic tumors⁴⁷¹.

An intraperitoneal dose once a week for 4-7 months in hamsters induced squamous cell papillomas of the trachea, epithelial papillomas, carcinomas, and neuroepithelial tumors of the nasal cavity, squamous cell papillomas of the bronchi and hepatic carcinomas^{472,473}.

Thorgeirsson et al. (1994) also gave NDEA intraperitoneally to 14 prosimian bushbabies²²⁴. After 10 years, 10 of the 14 have developed mucoepidermoid carcinoma of the nasal cavity and 2 developed liver carcinomas. Average cumulative dose was 0.75 g and the average latency period was ~ 22 months²²⁴. NDEA given orally induced liver tumors in 14 of 15 guinea pigs²²¹. Most tumors were carcinomatous or adenocarcinomatous. The tumors invaded lymphatic and blood vessels and metastasized to the lymph nodes, omentum, lung, kidney, and adrenal²²¹.

In a high profile publication in the journal *Science*, NDEA was reported to induce a dose-response of liver tumors (hepatocellular carcinoma) in rats: 92% of rats died from the liver tumors²¹⁸. Liver tumors are triggered by NDEA in dogs, monkeys, fish, parakeets, and pigs²²⁰. NDEA can stimulate the liver tumor marker called alpha-fetoprotein (AFP) produced by regenerating hepatic tumors^{341,347,349,474}.

NDEA was administered to mice intraperitoneally over 8 weeks^{356,357}. In the NDEA group, 14/16 (87.50% tumor incidence) mice had nodules visible on the liver surface³⁵⁵. The chemopreventive response of anti-cancer drugs to NDEA-induced cancer has been extensively tested. The tumor incidence was found to be 45.45% in the mice of the treated (lycopene extract - LycT) and NDEA group, which clearly demonstrated that LycT reduced the tumor incidence by 42%³⁵⁵. The average number of tumors per mouse was 5.74 ± 6.11 in the NDEA group, which was reduced to 2.32 ± 2.63 in the mice of the LycT + NDEA group. Histopathological assessment demonstrated that 82% of the tumors in the NDEA group had undifferentiated to poorly differentiated cells by 24 weeks. The tumors in the NDEA group were histologically classified as

poorly differentiated to undifferentiated HCC with small and large lesions of hyperchromatic cells with scanty cytoplasm³⁵⁵. Moreover, poorly to undifferentiated HCC are considered to have higher metastasizing potential than well-differentiated HCC⁴⁷⁵.

Lung/Respiratory Tract Cancer

Lung tumors have been observed in Syrian golden hamsters upon administration of NDEA by gavage or inhalation (Magee et al., 1976). Mice studies of oral administration of NDEA with an increased incidence of lung adenomas has been observed^{97,217,256,476}. The site and histological type of the tumors depended to certain extent on the mouse strain used. Tumor frequency was usually very high, approaching in many cases 100% in mice. Support of a dose-response relationship has been reported by Clapp *et al* (1970)^{97,217}.

In a hamster study, malignant liver cell tumors and tumors of the nasal cavity and bronchi were also induced via oral administration of NDEA⁴⁶⁰.

Groups of 20 male and 20 female 8-week-old Swiss mice were treated with a single subcutaneous injection of 0, 2, 4, 8, 16, or 32 mg/kg body weight of NDEA. In treated mice, the incidences of lung tumors (including adenomas and carcinomas) were 16/39, 18/38, 24/39, 25/39, and 21/40, compared to 33/218 in controls; 3 treated mice developed subcutaneous sarcomas with an average survival of 88 weeks²⁹⁹.

Treatment of newborn mice with a single subcutaneous dose of NDEA of 50 mg/kg bw caused a significant increase in the number of lung adenomas. Most of the animals also developed hepatomas within 6 months⁴⁷⁷. When groups of 40 or 120 gerbils were given once-week subcutaneous injections of NDEA, high incidences (66-80%) of multifocal tumors of the nasal cavity were observed. In addition, papillomas of the tracheobronchial system, adenomas, and

carcinomas of the lung, as well as cholangiocellular and hepatocellular carcinomas were seen^{298,299}.

Subcutaneous administration of NDEA to Algerian hedgehogs caused benign and malignant tumors of the liver and lung⁴⁷⁰. NDEA has had extensive use as a carcinogen in experimental animal model systems such as inducing lung tumors in mice after only 3 doses only 4 weeks apart⁴⁷⁸. NDEA is used to study nutritional and dietary effects of fat modulation on cancer such as in NDEA-induced lung cancers⁴⁷⁸.

The carcinogenic effects of a single dose of diethylnitrosamine (DEN-a former name of NDEA and the same chemical) were studied in three inbred strains of mice⁴⁷⁹. The most predominant tumors observed were lung adenomas, leukemia, and liver tumors⁴⁷⁹. Mice of strain AKR/J developed both leukemia and lung tumors; SWR/J mice were most susceptible to lung tumor development; and in C57BL/6J mice liver lesions including liver tumors occurred⁴⁷⁹. Studies on NDEA-induced liver tumors in mice demonstrated metastatic foci in the lungs in 22%⁴⁸⁰ and 38% of the animals. An intraperitoneal dose once a week for 4-7 months in hamsters induced squamous cell papillomas of the trachea, epithelial papillomas, carcinomas, and neuroepithelial tumors of the nasal cavity, squamous cell papillomas of the bronchi and hepatic carcinomas^{472,473}.

Administration of NDEA via three separate approaches (e.g., tube feeding, inhalation, and subcutaneous injection) caused cancer including squamous cell carcinoma of the trachea. In another study within 78 weeks after first exposure to NDEA, 39 out of 56 hamsters treated with NDEA developed tumors⁴⁸¹. Primarily, neoplasms of the nasal cavity and tracheal tumors were observed, as well as a few hepatocellular adenomas and sarcomas at the injection site⁴⁸¹. NDEA is carcinogenic in Syrian golden hamsters, primarily to the nasal cavity and trachea⁴⁸¹.

NDEA treatment of c-Myc transgenic mice significantly accelerated tumor growth and caused metastatic spread of HCC to lung but this treatment also induced primary lung cancer growth⁴⁸². The c-Myc transgenic model can be used in a short-term cancer bioassay with the genotoxic agent NDEA. Lung cancer was induced in mice with NDEA as tumors were detected in 46.8% of mice provided with 100 ppm NDEA given orally in drinking water³⁵⁴. Characteristic features of these lung tumors are: (i) appearance of tumors within a short period of time i.e. less than 75 days³⁵⁴. Expression of oncoproteins, c-myc and c-jun, was detected in all lung tumors.

Tracheal tumors have been observed in Syrian golden hamsters upon administration of NDEA by gavage or inhalation (Magee et al., 1976). NDEA causes tumors in the trachea of snakes which dies from these tumors (Figure 4)³⁷⁶. Fourteen snakes of the species *Python reticulatus* were randomized after one year's adaptation in the laboratory, i.e., at the age of 18 months³⁷⁶. The NDEA-containing aqueous solution was administered by oral gavage. Five untreated animals served as controls. Snakes receiving higher doses of NDEA died from toxic liver and kidney damage within the first year of experimentation. The three snakes receiving NDEA died within the last three months of the second year of treatment³⁷⁶. These animals had developed multiple benign and malignant tumors in the liver and the kidney. The two animals that died last also developed tumors in the oral cavity and the trachea³⁷⁶. Animals treated with NDEA died from tumors in the trachea.

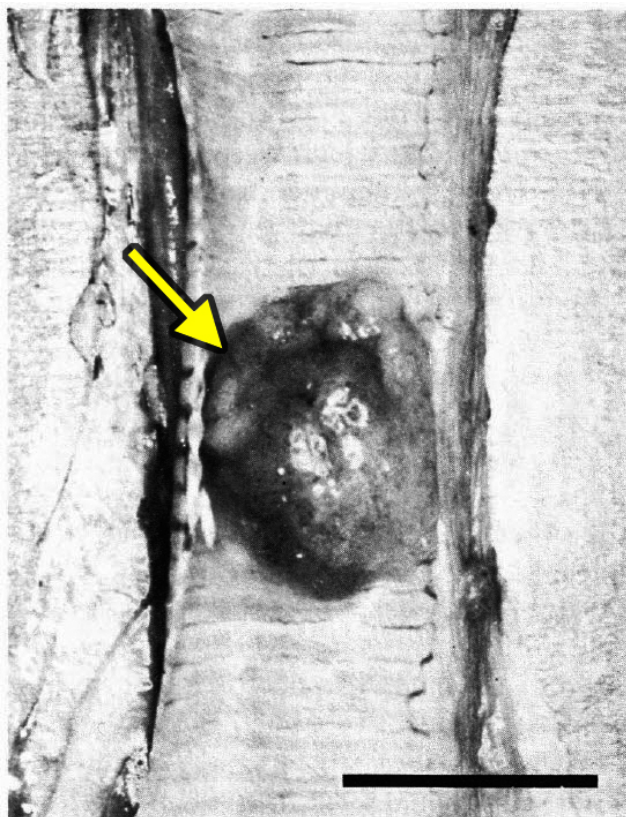


Figure 5. NDEA-induced papillary adenoma in the trachea of a snake (*Python reticularis*)³⁷⁶.

A positive-dose response for tumor induction from NDEA in the upper respiratory tract was observed after 12 weekly subcutaneous doses of 0.5, 1, 2 or 45 mg/animal; tumor yields in the nasal cavity and the larynx ranged from 17-72% and in the trachea from 88-100%; one liver tumor was observed²²⁰. There was not a threshold (no observed effect level – NOEL) for fatal liver tumors in female Fischer rats; a low dose of 0.45 ppm in the drinking water produced esophageal tumors after a 30-week exposure²²².

Gastric Cancer

NDEA induces tumors of the forestomach in rats²²⁰. In mice studies given oral administration of NDEA, squamous-cell carcinomas of the esophagus and forestomach were found^{97,217,256}. Tumors of forestomach and esophagus developed in all of the 20 male and female inbred Chinese hamsters exposed to NDEA in the drinking water for 17-26 weeks. Squamous cell

carcinomas accounted for 23% of the stomach tumors and the 15% of the esophagus as well as hepatocellular carcinomas in hamsters by oral NDEA⁴⁸³.

In 3 groups of 40 Chinese hamsters, subcutaneous NDEA treatment once a week produced squamous cell papillomas of the cheek pouch, tongue, pharynx, esophagus, and forestomach in up to 100% animals. NDEA also caused carcinomas in these animals⁴⁸⁴.

In Lijinsky et al rats were exposed to NDEA in drinking water at various concentrations and exposure durations²²². Animals were allowed to die with tumors, and time to death was used as one measure of NDEA potency. Time to death was inversely related to dose so animals exposed to a higher dose died more quickly²²². Dose-related tumors were found in the liver and upper gastrointestinal tract, including the esophagus, forestomach, tongue and oropharynx²²²

Blood Cancers (e.g., leukemia)

In a *human* lymphoblastoma cell line, CYP2A3 was more effective than CYP2E1 in activating NDEA³⁹⁵. This supports that NDEA can be activated in human tissues. The carcinogenic effects of a single dose of NDEA were studied in three inbred strains of mice⁴⁷⁹. The most predominant tumors included leukemia⁴⁷⁹. Mice of strain AKR/J developed leukemia⁴⁷⁹. NDEA in the tank water induced tumors of the hematopoietic (blood) system and hepatocellular carcinomas and adenomas in 41/94 exposed frogs⁷⁶.

Esophageal Cancer

Swenberg et. al, 1991⁶³ found it was likely that P450 isozymes other than CYP2E1 are responsible for *human* esophageal activation of NDEA. Another study found that rat esophagus contains CYP2A3 and/ or CYP2A6 similar to humans, important isozymes in activating NDEA³⁹⁵. In mice studies given oral administration of NDEA, they developed squamous-cell carcinomas of the esophagus^{97,217,256}. Tumors of the esophagus developed in all of the 20 male and female inbred

Chinese hamsters exposed to NDEA in the drinking water for 17-26 weeks. Squamous cell carcinomas accounted for 15% of the esophagus in hamsters by oral NDEA⁴⁸³. NDEA is shown to induce esophageal tumors in rats²²⁰. NDEA also induced nasal tumors, a related head and neck tumor²²⁰. Another rat study found carcinomas of the esophagus were induced in 9/14 male and 5/14 female 12-week-old Buffalo rats after feeding NDEA in the diet for 26 weeks⁴⁸⁵. In the previously reported study by Lijinsky et al rats were exposed to NDEA in drinking water at various concentrations and exposure durations²²². Animals were allowed to die with tumors, and time to death was used as one measure of NDEA potency. Time to death was inversely related to dose so animals exposed to a higher dose died more quickly²²². Dose-related tumors were found in the liver and upper gastrointestinal tract, including the esophagus, forestomach, tongue and oropharynx²²²

There was not a threshold (no observed effect level – NOEL) for fatal liver tumors in female Fischer rats and a low dose of 0.45 ppm in the drinking water produced esophageal tumors after a 30-week exposure²²². In 3 groups of 40 Chinese hamsters, subcutaneous NDEA treatment once a week produced squamous cell papillomas of the cheek pouch, tongue, pharynx, esophagus, and forestomach in up to 100% animals. NDEA also caused carcinomas in these animals⁴⁸⁴

NDEA induces tumors of the forestomach in rats²²⁰. In mice studies given oral administration of NDEA, squamous-cell carcinomas of the esophagus and forestomach were found^{97,217,256}. Tumors of forestomach and esophagus developed in all of the 20 male and female inbred Chinese hamsters exposed to NDEA in the drinking water for 17-26 weeks. Squamous cell carcinomas accounted for 23% of the stomach tumors and the 15% of the esophagus as well as hepatocellular carcinomas in hamsters by oral NDEA⁴⁸³.

Kidney Cancer

NDEA induces kidney tumors in rats²²⁰. Mohr and Hilfrich reported a single dose of NDEA induced kidney tumors³⁵². NDEA for 240 days caused induced adenomas of the kidney in 4 rats⁴⁸¹.

In the previously discussed study by Thorgerirsson et al. (1994) kidney tumors were found with other types when they gave NDEA intraperitoneally to 14 prosimian bushbabies²²⁴. Average cumulative dose was 0.75 g and the average latency period was ~ 22 months²²⁴. NDEA given orally induced liver tumors in 14 of 15 guinea pigs²²¹. Most tumors were carcinomatous or adenocarcinomatous. The tumors invaded lymphatic and blood vessels and metastasized to the lymph nodes, omentum, lung, kidney, and adrenal²²¹

NDEA and Dose-Response

There has been one dietary study, *Zheng*, that quantified NDMA and NDEA exposure, and risk of pancreatic cancer²³⁴. The study reported 89% statistically significant increase in risk of pancreatic cancer associated with NDEA intake (OR= 1.89, 95% CI: 1.41-2.53) for Quartile 3 and 128% statistically significant increase in risk of pancreatic cancer (OR= 2.28, 95% CI: 1.71-3.04). The fourth quartile of exposure to NDEA lower bound was defined 90 nanograms per day/1000kcal so for a diet of 2,000 kcalories that would equal 180 nanograms per day. If this was converted to a lifetime exposure of 60 years, it would equate to 3,942 micrograms of NDEA. By way of comparison, one of the highest levels reported in the drugs containing valsartan of NDEA was a Torrent at 16.93 ppm. This would equate to 5,417 nanograms in one 320 mg tablet. A patient taking these highly NDEA contaminated tablets would reach this cumulative dose in 2 years.

However, as expressed earlier, while this is an example of cumulative NDEA exposures that resulted in a statistically significant increased risk of cancer, it should not be considered as bright line threshold that is necessary to meet in order for there to be a causal relationship between

NDEA exposure and cancer response. It is well understood that individuals show widely variable susceptibility to carcinogenic factors, and the dose-response curve is in fact a reflection of the tolerance distribution. Each modulating factor divides the population up into subpopulations of different susceptibility so that nonlinearities that could be present in a homogeneous population are flattened out⁴⁸⁶.

Bradford Hill Analysis for NDEA

The Bradford Hill criteria are the following: strength of association, consistency across populations, specificity, temporality, dose-response (biologic gradient), plausibility, coherence, experiment, and analogy³⁹. These criteria as applied to whether the NDEA in valsartan containing drugs can cause human cancer can generally be described as follows:

1. Strength of association/Statistical significance. If the risk of developing cancer is higher in persons with more exposure to NDEA, then that increases the likelihood of causality and that the association is not due to chance alone. To evaluate strength of association, I reviewed the epidemiology studies, occupational and dietary, that quantified the amount of NDMA or NDEA exposure. I found these studies of greater value as they would be the best evidence of the relationship between NDMA and/or NDEA exposure and human cancer.

There has been one dietary study, *Zheng*, that quantified NDMA and NDEA exposure, and risk of pancreatic cancer. The study reported a 35% increased risk for Quartile 2 that did not rise to statistical significance (OR=1.35, 95% CI:1-1.82), 89% statistically significant increase in risk of pancreatic cancer (OR= 1.89, 95% CI: 1.41-2.53) for Quartile 3 and 128% statistically significant increase in risk of pancreatic cancer (OR= 2.28, 95% CI: 1.71-3.04).

Zheng strongly supports this Bradford Hill (BH) factor of causation given the statistically significant increase seen in the more exposed groups (second, third and fourth quartiles) as

compared to the lesser exposed group (first quartile). I place significant weight on this factor as statistically significant increased risk strengthens casual association.

- 2. Consistency of the association.** A consistent association would be one that has been repeatedly observed in various populations, places, circumstances, and times. This criteria is meaningful when evaluating animal cancer studies as well as the available human epidemiology.

As set forth above, there have been numerous animal cancer studies that show a clear association between NDEA exposure and liver, lung, gastric, blood, esophageal and kidney cancers. Zheng shows a statistically significant association between NDEA exposure and pancreatic cancer. The Zheng study together with the consistency of the association between NDEA exposure and cancer across many mammalian species including mice, rats, hamsters, guinea pigs, rabbits, dogs, swine, monkeys, snakes, fish, mollusks, cats, gerbils, prosimian bushbabies and chickens, studied by several researchers, using different study designs, over several decades is significant and gives strong support in favor of a causal association.

- 3. Specificity of the association:** If exposure to a chemical causes only specific disease, then its causal link to that disease can be strengthened. However, Bradford Hill recognized that this criterion cannot be overemphasized as “one to one relationships are not frequent.” (Hill 1965) Carcinogens are known to often cause multiple types of cancer. For example, smoking is an accepted cause of multiple cancers. As such, this Bradford Hill criteria is less relevant for cancer causation analysis. Because of this, I gave this factor little weight in my causal analysis.

- 4. Temporality:** This factor is important in the causal analysis. In any study of scientific value to answer the question does NDEA cause human cancer, the exposure to NDEA must come

before the diagnosis of clinical cancer. In the studies relied upon, whether animal or human, the exposure to NDEA came before the diagnosis of cancer so this factor is satisfied.

5. Biologic gradient: This refers to whether there is a demonstrated dose-response. Does the risk of cancer increase with increasing amount of exposure? Exposure can be defined by amount or duration, or combination. If the risk of cancer does increase with exposure, then there is an increased likelihood of a causal relationship. However, one needs to keep in mind that with for genotoxins, like NDEA, there is no safe dose as each exposure has the potential to do permanent damage to the DNA which can cause cancer³⁰.

There has been one dietary study, *Zheng*, that quantified NDMA and NDEA exposure, and risk of pancreatic cancer. The study reported a 35% increased risk for Quartile 2 that did not rise to statistical significance (OR=1.35, 95% CI:1-1.82), 89% statistically significant increase in risk of pancreatic cancer (OR= 1.89, 95% CI: 1.41-2.53) for Quartile 3 and 128% statistically significant increase in risk of pancreatic cancer (OR= 2.28, 95% CI: 1.71-3.04). This increasing risk with increasing NDEA exposure is evidence of a classic dose-response.

Dose-response is also seen clearly in the animal studies. Some examples are: Peto et al (1991) demonstrating a classic dose-response between exposure to NDEA and the induction of tumors including esophagus in the rat^{19,74}. In the large dose-response study in Colworth rats, tumor incidence data were adjusted to account for the presence of two fatal tumor types, which occurred at high incidences¹⁹. At low doses, Peto et al reported the incidence of liver tumors was linear related to dose⁷⁴. The increased tumor-formation rate at doses > 1 ppm in the large bioassay¹⁹ corresponded to the increased NDEA-induced cell proliferation above 1 ppm in male rat liver⁴⁵². Above 1 ppm NDEA in drinking water, cell proliferation increased in a time- and dose-related manner, which correlated with the increased tumor incidence seen above 1 ppm in male rat liver¹⁹.

Consequently, tumor incidence in the liver was related to both adduct formation and cell proliferation rates. The dose-responses for liver and esophageal tumors showed that incidence was exponentially related to dose where tumors were found. Lifetime exposure of BDII rats to NDEA in drinking water at doses increasing from 0.7 to 132.5 ppm produced dose-related decreases in time to tumor (tumor latency) for both liver and esophagus tumors^{202,315}.

Dose-response for NDEA-induced liver-cell proliferation was also studied by Deal et al. (1989) using 6-week-old male F344 rats exposed to NDEA in drinking water for 1, 4 or 10 weeks. Exposure to NDEA increased cell proliferation by 300% and 400% in all lobes. The minimum dose required for enhanced cell proliferation correlated with the 1 ppm dose in male rat liver, where there was an increase in dose-response at high-dose exposure compared with low-dose exposure⁴⁵².

The Zheng study provides strong evidence of NDEA dose-response and strongly supports this Bradford Hill (BH) factor of causation given the statistically significant increase seen in the more exposed groups (second, third and fourth quartiles) as compared to the lesser exposed group (first quartile). The NDEA animal cancer studies lend further support by demonstrating a classis dose-response. I place significant weight on this factor as strong evidence of dose-response strengthens casual association.

6. Plausibility: Is the association between cancer and NDEA biologically plausible? Mechanistic evidence can add biological plausibility to epidemiological findings which strengthens causal inference. In evaluating experimental animal studies, mechanistic studies can provide valuable data to address the similar response between experimental animals and humans. This helps to identify the mechanisms contributing to the induction of the observed animal tumors from carcinogens and to determine whether analogous mechanisms may be operative in

humans. While biologic plausibility does not require proof of mechanism, the mechanisms in which NDEA causes cancer was extensively addressed in the discussion of NDEA possessing 9 out of the 10 key characteristics of a carcinogen. There are also the following studies demonstrating NDEA activation by human blood (Crespi et al., 1990) and human liver microsomes activating NDEA (Yamazaki et al. (1992a) as well as animal model esophageal activation of NDEA (Swenberg et al., 1991); (Crespi et al., 1990) so I assigned great weight to this factor in my analysis.

7. Coherence: NDEA induced cancer is consistent with the generally known facts and the biology of cancer. The natural history and biology of cancer tells us that cancer initiation and promotion result from exposure to chemicals like NDEA that can be metabolically activated to electrophiles; are genotoxic; induce genomic instability; alter DNA repair; induce epigenetic alterations, oxidative stress, and chronic inflammation; are immunosuppressive; cause immortalization and alter cell proliferation, cell death and nutrient supply (key characteristics of carcinogenesis). Since NDEA produced tumors in every animal species and strain in which it was investigated, and since the target organs were species-specific, it is likely that NDEA activation is dependent upon bioavailability of relevant P450 enzymes within the involved organ. Since the P450 enzymes are expressed in many tissues, it is likely that the bioavailability of NDEA is similar to NDMA. In humans, a large percentage of ingested NDMA is available extrahepatically (outside the liver). The extrahepatic availability of NDMA ranges from 49% to 93% in large mammals. This is most likely similar for NDEA. Once extrahepatic, NDEA would enter the systemic circulation which provides a functional blood supply to all blood tissues throughout the body. Cytochrome P450 enzymes, which metabolize NDEA into its carcinogenic metabolite, are expressed throughout the body so it makes biological sense that the liver, lung,

stomach, esophagus, kidney, blood, pancreas, bladder, colorectal and prostate would have an increased risk for cancer development after ingestion of NDEA, such as taking contaminated valsartan.

8. Experiment: As NDEA is a known carcinogen, it would be unethical to conduct human randomized control trials (RCTs) so there is no experimental human data. For this reason, NDEA has been largely tested in animal models, and human tissues and cells all of which support a finding of causation between NDEA and human cancer. While I would normally give significant weight to RCTs, because of the impossibilities in conducting NDEA RCTs in humans, I attribute less weight to this factor, even though non-human models and human in vitro studies provide significant support.

9. Analogy: Bradford Hill states that in some circumstances it would be fair to judge by analogy. For example, in this case, N-nitroso compounds, which includes NDEA, are known to display extremely high carcinogenic potency. Nitrosamines all have the similar generic chemical structure with the essential feature being the N–N=O structure. The Report on Carcinogens, 14th Edition of the National Toxicology Program, Department of Health and Human Services lists all the following 15 N-Nitrosamines as reasonably anticipated to be a human carcinogens: N-Methyl-N'-nitro-N-nitrosoguanidine, N-Nitrosodi-n-butylamine, N-Nitrosodiethanolamine, N-Nitrosodiethylamine, N-Nitrosodimethylamine, N-Nitrosodi-n-propylamine, N-Nitroso-N-ethylurea, 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone, N-Nitroso-N-methylurea, N-Nitrosomethylvinylamine, N-Nitrosomorpholine, N-Nitrosonornicotine, N-Nitrosopiperidine, N-Nitrosopyrrolidine, and N-Nitrososarcosine. An analogy can be drawn regarding the carcinogenic nature of NDEA as it is in the N-Nitrosamines class of chemical compounds which are all considered to be reasonably anticipated human carcinogens. One of these related N-nitroso

compounds is NDMA which for reasons previously set forth in this report has been shown to be a human carcinogen. The class of N-nitroso compounds is known to be carcinogenic which lends support to a finding a causal relationship between NDEA exposure and human cancer. I assigned moderate weight to this factor.

It is my opinion, stated with a reasonable degree of medical and scientific certainty, based on a totality of the evidence, which includes the Zheng study, NDEA animal cancer studies, animal and human tissue studies, that NDEA in the valsartan containing drugs increases the risk of and causes human liver, lung, stomach, esophagus, kidney, blood, pancreas, bladder, colorectal and prostate cancer.

LATENCY

NDMA REDUCES TUMOR LATENCY

NDMA initiates cancer and dramatically shortens tumor latency. The latency period is defined by the National Cancer Institute as “the time that passes between being exposed to something that can cause the disease (such as radiation or a virus or a carcinogen) and having symptoms”. *Cancer latency* is the time period from which a cancer starts until it is diagnosed. Cancer latency can be estimated from the natural growth rate of cancers after exposure to NDMA and development of symptoms or death from the NDMA-induced cancers. The studies for NDMA-induced tumors in experimental animals demonstrate latency as early as 10 weeks to a year. NDMA reduces tumor latency by acting as a tumor initiator and tumor promoter via 9 of the 10 key characteristics of carcinogens.

As will be discussed below, patients who develop cancers from contaminated valsartan are at elevated risk for cancer spread and recurrence once their cancer is removed by surgery and/or treated with chemotherapy and radiation. Cancer may recur after removal at any time and most

cancer patients die from cancer recurrence or spread (called metastasis) of their cancer. Thus, cancer patients exposed to contaminated valsartan will require life-long surveillance to monitor for cancer recurrence and spread of their cancer. There can be a tumor dormancy and micrometastatic state with slow growing disease in patients who have their cancer surgically removed. Cancer studies show that malignant cells that disseminate early can reside as single cells or as micrometastatic clusters in the bone marrow^{487,488}. These disseminated tumor cells lack the ability to colonize or are prevented from colonizing by the environment, resulting in a state of proliferative dormancy⁴⁸⁹.

A clear strategy to detect cancer after cancer surgery is challenging involving weighing risks/benefits of cancer screening and not always possible. Thus, close lifelong follow-up is recommended for cancer patients exposed to valsartan contaminated NDMA. Many cancer patients do not die from their primary tumor but from metastasis or recurrent tumors⁴⁹⁰.

Because NDMA acts as both a tumor initiator and tumor promoter, cancers caused by this chemical do not necessarily take years or decades to develop. Many studies now confirm the long-held notion that metastases emerge from dormant tumor cells that have disseminated early but remain dormant because they lack the proper permissive or causative factors to grow into macroscopic tumor^{4,9,491-498}.

NDMA reduces latency as a cancer initiator and cancer promoter

In Magee and Barnes (1956), NDMA administered in the diet induced liver tumors and metastases *between 26 and 42 weeks* in 19 of 20 animals⁸⁹. In Magee and Barnes (1962), NDMA administered in the diet induced large kidney, liver, and lung tumors *after 26 weeks* in 13 of 18 rats⁹⁰. In some rats, a single oral dose of NDMA induced kidney tumors. The resemblance between the NDMA-induced kidney tumors in the rat and the kidney tumors in humans is very close⁹⁰. In

studies by Mohr et al (1974) in hamsters, NDMA induced various tumor types including malignant haemangioendotheliomata of the liver and kidney, hepatocellular carcinoma, squamous cell carcinoma, cholangiocellular carcinoma, stomach cancer, lymphoma, and metastasis to the lung after *13 to 28 weeks*²⁴⁷. In studies by Takayama et al (1963), NDMA in the diet for 5 months induced tumors within *121 to 190 days* in mice²⁵². In Terracini et al (1964) in a high profile publication in Nature, NDMA induced large kidney tumors (up to 8 cm), liver, and lymphoma tumors in rats *at 36 weeks* of age⁷⁵. In Zak et al (1959), NDMA induced kidney and lung tumors in rats between *111 and 160 days*²⁹⁵. In Swan et al (1980), NDMA induced kidney tumors in rats by *28-43 weeks*³⁰⁶. In Anderson et al (1986), NDMA induced liver and lung tumors by *16 to 28 weeks* in mice²⁴⁸. In Toth et al (1964), a single injection of NDMA induced tumors *after 141 days* in mice²⁴⁹. NDMA induced lung adenomas, hemangiomas, and hemangiosarcomas mainly in the liver, adenomas of kidney, hepatomas, and liver cell carcinomas²⁴⁹. In Otsuka et al (1971) NDMA administered in the diet induced lung (e.g. in 89/115), kidney, spleen, lung, soft tissue sarcomas, and hepatocellular tumors *after 5 months* in mice⁸¹.

In larger animals such as rabbits, Le Page et al (1969) demonstrated that NDMA induced liver tumors with metastases in the lung and a kidney tumor after 23 weeks⁹¹. In rats, a single dose of NDMA induced tumors in all the surviving rats after 8 to 11 months³⁰⁹. In guinea pigs NDMA induced liver tumors *after 6-49 weeks*⁹². In fish, NDMA induced liver tumors *after 9 to 20 months*²⁵⁹. Guppies developed liver tumors after *13 months* of exposure to NDMA²⁶⁰.

NDMA and NDEA initiate and promote cancer growth via similar mechanisms in animal and human tissues and cells via the 9 of the 10 key characteristics of carcinogens. This, NDMA and NDEA would act trigger cancer progression is a similar time frame in humans as in animals. In summary, NDMA stimulates many key characteristics including inflammation, angiogenesis,

oxidative stress, immunosuppressive behavior, cell proliferation, and cell death which act as tumor promoters to accelerate tumor growth. By stimulating genotoxicity and genomic instability, NDMA can initiate cancer. By stimulating inflammation and angiogenesis, NDMA can trigger activation of dormant micrometastases. NDMA can reduce tumor latency and promote tumor dormancy escape via genotoxic and non-genotoxic mechanisms. Thus, NDMA may initiate and stimulate tumor growth quickly (within months) via explosive tumor and metastatic growth from dormant cancers.

NDMA CAN ACTIVATE TUMOR DORMANCY

The view of cancer only resulting from genetic changes, mutagens or only direct effects on tumor cells is outdated as well as the assumption that cancer takes decades to develop. The latency period (from time of exposure to development of cancer) can occur much quicker (e.g., 6 months to a year) via non-genotoxic mechanisms such as stimulation of inflammation, angiogenesis, oxidative stress, and immunosuppression resulting in tumor dormancy escape^{4,9,11,439,499-501}. NDMA exhibits all of these characteristics so it can stimulate tumor dormancy escape. NDMA is not required to be a “tumor initiator” and can act as a “tumor promoter” to activate dormant cancers. This is a biologically plausible mechanism to support carcinogenesis with short-term exposure of NDMA and NDEA. As a result, cancers caused by these carcinogens do not necessarily take years or decades to develop.

Cancer cells from a primary tumor can disseminate to other tissues, remaining dormant and clinically undetectable for many years. Over 30% of healthy individuals harbor microscopic dormant cancers¹. While many healthy people have dormant cancerous tumors, only a minority will develop cancer⁵⁰². Dormancy is a stage in cancer progression where the cancer cells are not dividing but survive in a quiescent state, sometimes indefinitely. Dormant tumors can remain

indefinitely but can serve a cancer initiator if exposed to a trigger such as carcinogens like NDMA or NDEA.

Under certain environmental conditions, these dormant cells escape dormancy and begin proliferating again. “Stressful” events such exposure to a carcinogen, wounding, surgery, biopsy, or chemotherapy can trigger tumor dormancy escape^{9,500}. Dormant tumors have been identified at autopsy in normal adults who died of trauma and without prior history of clinical evidence of cancer, including 39% for *in situ* breast carcinoma, 46% for *in situ* prostate cancer, and 36% for thyroid carcinoma in people aged 50–70^{1,499,503}.

Microscopic human cancers can remain in an asymptomatic, non-detectable, and occult state for the life of a person or animal⁴⁹⁹. Clinical and experimental evidence indicate that human tumors can persist for long periods of time as microscopic lesions that are in a state of dormancy (i.e., not expanding in tumor mass). Solitary, dormant cells persist in various tissues and large numbers of disseminated single cells may persist for extended time periods as a source of tumor dormancy^{504,505}. Tumors can present shortly after an accidental trauma in otherwise healthy individuals⁵⁰⁶.

Because it is well-established that tumor growth beyond the size of 1-2 mm is angiogenesis-dependent, large tumors may result from stimulated angiogenesis in otherwise microscopic, dormant tumors^{499,507}. Thus, tumor dormancy escape is critically dependent on the induction of angiogenesis⁵⁰⁸⁻⁵¹¹. There is scientific evidence that dormant tumors and dormancy escape can also occur in other tumor types such as colorectal, liver, kidney, bladder, gastric, blood (hematological) and pancreatic cancers⁵¹²⁻⁵²¹.

Inflammation is a hallmark of cancer^{33,101,184,522,523} and is essential for cancer growth by oncogenes in a genetically engineered cancer model¹⁸². Inflammation triggers escape from

latency¹⁸⁷. Chronic Inflammation (key characteristic #6) is associated with tumor-promoting activity and can enable tumor cells to escape cancer dormancy or tumor latency^{33,187}. For example, sustained lung inflammation caused by tobacco smoke exposure converted disseminated, dormant cancer cells to aggressively growing metastases⁵²¹. Chronic inflammation induces the formation of neutrophil extracellular traps (NETs), which can awaken dormant cancers⁵²¹.

Chronic inflammation can trigger dormant cells to develop into growing tumors that can spread throughout the body^{187,524}. Carcinogen-induced chronic inflammation in the tumor microenvironment via aberrant pro-inflammatory cytokine release can enable cancer progression¹¹. Inflammation can act as a co-initiator of tumor growth^{11,182,183}. Inflammation caused by carcinogens, wounding, surgery, chemotherapy, and radiation can trigger activation of dormant micrometastases^{9,500}. Chronic inflammation and inflammation-induced cell proliferation, oxidative stress, and reactive chemical species (e.g., ROS - reactive oxygen species) stimulate DNA damage and inhibit DNA repair to drive mutations and tumor progression^{171,177,525}. Thus, there is a vicious protumorigenic feedback loop between apoptosis, inflammation, DNA damage, and carcinogenesis¹¹.

The majority of cancer deaths (60% to 90%) are caused by metastases, which is the spread of cancer throughout the body⁵²⁶. Inflammation in the tumor microenvironment can promote and trigger the escape of metastatic tumor latency^{187,527}. NDMA-stimulated chronic inflammation can similarly ignite the escape of metastatic tumor latency and dormancy escape.

NDMA can activate occult or dormant tumors to become invasive cancer in humans. Scientific studies show that as a tumor promoter, NDMA could cause tumors with short exposure durations, and tumors observed after short term exposure are indeed biologically plausible and should not be disregarded.

LATENCY

NDMA and NDEA initiate cancer and dramatically shorten tumor latency. The latency period is defined by the National Cancer Institute as “the time that passes between being exposed to something that can cause the disease (such as radiation or a virus or a carcinogen) and having symptoms”. *Cancer latency* is also defined as the time-period from which a cancer starts until it is diagnosed. Cancer latency can be estimated from the natural growth rate of cancers after exposure to NDMA/NDEA and development of symptoms or death from NDMA and/or NDEA-induced cancers. The latency studies for NDMA or NDEA-induced tumors in experimental animals demonstrate latency as early as 10 weeks to a year. NDMA and NDEA also reduce tumor latency by acting as tumor-promoter exhibiting 9 of the 10 key characteristics.

NDMA and NDEA reduce tumor latency

Because NDMA and NDEA act as both tumor initiators and tumor promoters, cancers caused by these chemicals do not necessarily take years or decades to develop. Cancer initiators such as nitrosamines, e.g., NDMA and NDEA, can cause an irreversible genetic modification in a normal cell, which primes the cell for uncontrolled growth. Initiators either bind to and change a cell's DNA, or exert their effect through more indirect, epigenetic changes. NDMA and NDEA may then activate occult or dormant tumors to become invasive cancer in humans. Scientific studies show that in their capacity as tumor promoters, NDMA and NDEA can cause tumors with short exposure durations, and tumors observed after short term use are indeed biologically plausible. As set forth in detail previously, NDMA and NDEA both exhibit 9 of the 10 key characteristics of a carcinogen so they each have over 4 key characteristics that promote cancer in addition to both being cancer initiators.

NDMA as a cancer initiator can exhibit shortened latency periods

The cancer animal studies demonstrate short latency periods for NDMA-induced cancer. In Magee and Barnes (1956), NDMA administered in the diet induced liver tumors and metastases *between 26 and 42 weeks* in 19 of 20 animals⁸⁹. In Magee and Barnes (1962), NDMA administered in the diet induced large kidney, liver, and lung tumors *after 26 weeks* in 13 of 18 rats⁹⁰. In some rats, a single oral dose of NDMA induced kidney tumors. The resemblance between the NDMA-induced kidney tumors in the rat and the kidney tumors in humans is very close⁹⁰. In studies by Mohr et al (1974) in hamsters, NDMA induced various tumor types including malignant haemangioendotheliomata of the liver and kidney, hepatocellular carcinoma, squamous cell carcinoma, cholangiocellular carcinoma, stomach cancer, lymphoma, and metastasis to the lung *after 13 to 28 weeks*²⁴⁷. In studies by Takayama et al (1963), NDMA in the diet for 5 months induced tumors within *121 to 190 days* in mice²⁵². In Terracini et al (1964) in a high profile publication in Nature, NDMA induced large kidney tumors (up to 8 cm), liver, and lymphoma tumors in rats *at 36 weeks* of age⁷⁵. In Zak et al (1959), NDMA induced kidney and lung tumors in rats *between 111 and 160 days*²⁹⁵. In Swan et al (1980), NDMA induced kidney tumors in rats *by 28-43 weeks*³⁰⁶. In Anderson et al (1986), NDMA induced liver and lung tumors *by 16 to 28 weeks* in mice²⁴⁸. In Toth et al (1964), a single injection of NDMA induced tumors *after 141 days* in mice²⁴⁹. NDMA induced lung adenomas, hemangiomas, and hemangiosarcomas mainly in the liver, adenomas of kidney, hepatomas, and liver cell carcinomas²⁴⁹. In Otsuka et al (1971) NDMA administered in the diet induced lung (e.g. in 89/115), kidney, spleen, lung, soft tissue sarcomas, and hepatocellular tumors *after 5 months* in mice⁸¹.

In larger animals such as rabbits, Le Page et al (1969) demonstrated that NDMA induced liver tumors with metastases in the lung and a kidney tumor *after 23 weeks*⁹¹. In rats, a single dose

of NDMA induced tumors in all the surviving rats after 8 to 11 months³⁰⁹. In guinea pigs NDMA induced liver tumors *after 6-49 weeks*⁹². In fish, NDMA induced liver tumors *after 9 to 20 months*²⁵⁹. Guppies developed liver tumors after *13 months* of exposure to NDMA²⁶⁰.

Because NDMA exerts its carcinogenicity through 9 of 10 key characteristics, cancers caused by this carcinogen do not necessarily take years or decades to develop. In certain cases, the period of exposure to NDMA prior to cancer causation was relatively short (i.e., 10 to 36 weeks)⁷⁵. There is a longer latency period (more than 20 weeks) for the development of carcinoma in the fetal human pancreas explants with their relatively higher proliferative activity, compared with that of the adult human pancreas explants (10 to 12 weeks) with little or no mitotic activity. This suggests cumulative lifelong alterations in the adult pancreas induced by environmental chemicals which increase its susceptibility to nitrosamine. Thus, NDMA may reduce tumor latency via stimulating inflammation in the tumor microenvironment.

In practicality, NDMA induced tumor latency is realistically shorter than the above time periods as the studies did not detect the NDMA-induced tumor until the symptoms or death occur and are observed.

NDEA as a cancer initiator can have short latency periods

Animal studies provide some insight into the shortened latency periods associated with NDEA. For example, hepatic cell carcinoma in monkeys was induced within 27 months (earliest tumor 14 months, median time to tumor formation, 23 months) in 5 of 11 macaque monkeys including metastases to the lung⁵⁰. Lifetime exposure of BDII rats to NDEA in drinking water at doses increasing from 0.7 to 132.5 ppm produced dose-related decreases in time to tumor (tumor latency) for both liver and esophagus tumors^{202,315}. Thorgeirsson et al. (1994) also administered NDEA

intraperitoneally to 14 prosimian bushbabies²²⁴. The average latency period was ~ 22 months²²⁴

In a scientific publication in the high impact journal Science, 92% of rats given NDEA developed hepatocellular carcinoma in a narrow and highly reproducible time interval²¹⁸. A single dose of NDEA produced irreversible carcinogenic effects at 82 days²¹⁸. In studies by Stanton et al (1965); NDEA induced tumors *between the 10th and 30th week* after exposure to NDMA in fish⁵²⁸. In another study in guinea pigs, hepatocellular carcinoma was the main tumor-type induced in 14/15 animals *between 16 and 23 weeks*²²¹. Tumors occurred in the liver of a guinea pig that died *as early as 16 weeks* after the feeding commenced²²¹. All but 1 guinea pig that survived *over 23 weeks* of NDEA feeding developed advanced hepatic carcinomas.

Invasion of lymphatic and blood vessels was common as widespread extrahepatic (outside the liver) metastases were found in 3 animals including spread to lymph nodes, omentum, lung, kidney and adrenals²²¹. Seven guinea pigs surviving 40 weeks of treatment also developed lung tumors²²¹. The histological appearance of the liver and lung tumors in guinea pigs was similar to that described in rats²²¹. Characteristic features of these lung tumors in mice are: appearance of tumors within a short period of time i.e. *less than 75 days*³⁵⁴. Lung cancer was induced in mice with NDEA as tumors were detected in 46.8% of mice provided with 100 ppm NDEA given orally in drinking water³⁵⁴.

NDMA and NDEA as cancer promoters reduce latency periods

Many studies now confirm the long-held notion that metastases emerge from dormant tumor cells that have disseminated early but remain dormant because they lack the proper permissive or causative factors to grow into macroscopic tumor^{4,9,491-498}. Both NDMA and NDMA

act as cancer promoters by inducing oxidative stress, chronic inflammation, causing immunosuppression, cell immortalization, angiogenesis, proliferation and cell death. See Ten Key Characteristics of Carcinogens: NDMA and NDEA sections of this report.

By stimulating genotoxicity and genomic instability, NDMA and NDEA also can initiate cancer. By stimulating inflammation, oxidative stress, cell death, immunosuppression, and angiogenesis, NDMA and NDEA can trigger activation of dormant micrometastases. NDMA and NDEA can reduce tumor latency and promote tumor dormancy escape via non-genotoxic mechanisms. Thus, NDMA and NDEA may initiate and stimulate tumor growth quickly (within months) via explosive tumor and metastatic growth from dormant cancers.

Escape of dormant tumors can occur following exposure to a carcinogen such as NDMA or NDEA. Dormant tumors can be awakened by stimulation of inflammation and angiogenesis. For example, PPAR α activation triggers tumor dormancy escape via stimulating inflammation and angiogenesis³. In mice that lack PPAR α (PPAR α knockout mice), injected tumor cells fail to grow and stay dormant, recognizable as small nodules under the skin. Implantation of such dormant tumors into a wild-type mouse that has PPAR α activity triggers an explosive growth of the dormant tumor within 30 days³.

The trigger for tumor dormancy escape, as numerous animal studies have shown, is typically of non-genetic nature and can include: surgery, wounding at a site near or distant from the site of the occult tumor, sustained inflammation, or stimulated angiogenesis cells^{9,500,501}. A derivative of omega-6 fatty acids called epoxyeicosatrienoic acids (EETs), triggers an escape from tumor dormancy in mice carrying a primary dormant tumor by stimulating processes such as angiogenesis in the tumor microenvironment, as well as triggering the mushrooming of large numbers of metastatic tumors in multiple organs⁴. Stimulating angiogenesis in dormant tumors

triggered massive, unprecedented patterns of spread of cancer (called metastasis) and tumor dormancy escape⁴. Thus, dormant tumors can be awakened by non-genotoxic mechanisms such as tumor-promoting inflammation and/or angiogenesis via an explosive growth of the dormant tumor within 30-90 days³⁴⁷. Carcinogens can induce sustained inflammation, stimulated angiogenesis and pro-cancer growth factors such as pro-inflammatory cytokines or eicosanoids^{271,347}. Thus, there are biologically plausible mechanisms by which NDMA and NDEA cause cancer to switch from a microscopic to clinically detectable and invasive form in both animals and humans. These include genotoxic and non-genotoxic mechanisms such as inflammation, oxidative stress, and angiogenesis.

Moreover, long latency periods were viewed as a hallmark of cancer causation by genotoxic carcinogens, such as cigarette smoke. However, our understanding of the 10 key characteristics of carcinogens demonstrates that carcinogens can reduce tumor latency to 6 months as shown by our laboratory and others via stimulation of tumor-promoting processes in the tumor microenvironment such as inflammation and angiogenesis^{3,4,9}.

In addition to genotoxic mechanisms, NDMA and NDEA also exerts its carcinogenicity through the tumor microenvironment mechanisms. Thus, cancers caused by NDMA do not necessarily take years or decades to develop. Many studies now confirm that metastases emerge from dormant tumor cells that have disseminated early but remain dormant because they lack the proper permissive or causative factors to grow into macroscopic tumor. NDMA and NDEA can stimulate tumor dormancy escape within 6 months of exposure via non-genotoxic mechanisms including oxidative stress, inflammation, and angiogenesis.

NDMA and NDEA can also induce carcinogenesis in a short time-period through the cross talk between the tumor cells and the tumor microenvironment, including tumor-associated

macrophages. A tumor promoter using the nitrosamine (BBN model) of rat bladder carcinogenicity showed the occurrence of bladder tumors in rodents within a mere 5 months³⁴⁸. Thus, these studies provide sufficient evidence that carcinogens can act as a tumor promoter in the short term. In a parallel manner to these chemically induced bladder cancer models wherein a chemical (BBN, NDMA, or NDEA) acts as a tumor initiator, most people have dormant cancerous tumors. Thus, it is highly likely that many NDMA-treated patients already had dormant pre-cancerous lesions. People that have taken contaminated valsartan may have a long-lasting latency period as an occult (“hidden”) pre-tumor. This long-lasting occult stage occurs as “dormant” tumors await a cancer trigger such as NDMA or NDEA.

NDMA and NDEA can activate tumor dormancy

The view of cancer only resulting from genetic changes, mutagens or only direct effects on tumor cells is outdated as well as the assumption that cancer takes decades to develop. The latency period (from time of exposure to development of cancer) can occur much quicker (e.g., 6 months to a year) via non-genotoxic mechanisms such as stimulation of inflammation, angiogenesis, oxidative stress, and immunosuppression resulting in tumor dormancy escape⁴³⁹. NDMA exhibits all of these characteristics so it can stimulate tumor dormancy escape. NDMA is not required to be a “tumor initiator” and can act as a “tumor promoter” to activate dormant cancers. This is a biologically plausible mechanism to support carcinogenesis with short-term exposure of NDMA and NDEA. As a result, cancers caused by these carcinogens do not necessarily take years or decades to develop.

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will develop cancer⁵⁰². Dormancy is a stage in cancer progression where the cancer cells are not dividing but survive in a quiescent state, sometimes indefinitely. Dormant tumors can remain indefinitely but can serve as a cancer initiator if exposed to a trigger such as carcinogens like NDMA or NDEA.

Under certain environmental conditions, these dormant cells escape dormancy and begin proliferating again. “Stressful” events such exposure to a carcinogen, wounding, surgery, biopsy, or chemotherapy can trigger tumor dormancy escape^{9,500}. Dormant tumors have been identified at autopsy in normal adults who died of trauma and without prior history of clinical evidence of cancer, including 39% for *in situ* breast carcinoma, 46% for *in situ* prostate cancer, and 36% for thyroid carcinoma in people aged 50–70 years^{1,503}. Microscopic human cancers can remain in an asymptomatic, non-detectable, and occult state for the life of a person or animal⁴⁹⁹. Clinical and experimental evidence indicate that human tumors can persist for long periods of time as microscopic lesions that are in a state of dormancy (i.e., not expanding in tumor mass). Solitary, dormant cells persist in various tissues and large numbers of disseminated single cells may persist for extended time periods as a source of tumor dormancy^{504,505}. Tumors can present shortly after an accidental trauma in otherwise healthy individuals⁵⁰⁶. Because it is well-established that tumor growth beyond the size of 1-2 mm is angiogenesis-dependent, large tumors may result from stimulated angiogenesis in otherwise microscopic, dormant tumors^{499,507}. Thus, tumor dormancy escape is critically dependent on the induction of angiogenesis⁵⁰⁸⁻⁵¹¹. There is scientific evidence that dormant tumors and dormancy escape can also occur in other tumor types such as colorectal, liver, kidney, bladder, gastric, blood (hematological) and pancreatic cancers⁵¹²⁻⁵²¹.

NDMA can activate these occult or dormant tumors to become invasive cancer in humans. Scientific studies show that as a tumor promoter, NDMA could cause tumors with short exposure

durations, and tumors observed after short term exposure are indeed biologically plausible and should not be disregarded.

Inflammation is a hallmark of cancer^{33,101,184,522,523} and is essential for cancer growth by oncogenes in a genetically engineered cancer model¹⁸². Inflammation triggers escape from latency¹⁸⁷. Chronic Inflammation (key characteristic #6) is associated with tumor-promoting activity and can enable tumor cells to escape cancer dormancy or tumor latency^{33,187}. Sustained lung inflammation caused by tobacco smoke exposure converted disseminated, dormant cancer cells to aggressively growing metastases⁵²¹. Sustained inflammation induced the formation of neutrophil extracellular traps (NETs), which awakened the dormant cancer⁵²¹.

Chronic inflammation can awaken dormant cells to develop into growing tumors that can spread throughout the body^{187,524}. Carcinogen-induced chronic inflammation in the tumor microenvironment via aberrant pro-inflammatory cytokine release can enable cancer progression¹¹. Inflammation can act as a co-initiator of tumor growth.¹⁸³ Inflammation caused by carcinogens, wounding, surgery, chemotherapy, and radiation can trigger activation of dormant micrometastases^{9,500}. Chronic inflammation and inflammation-induced cell proliferation, oxidative stress, and reactive chemical species (e.g., ROS - reactive oxygen species) stimulate DNA damage and inhibit DNA repair to drive mutations and tumor progression^{171,177,525}. Thus, there is a vicious protumorigenic feedback loop between apoptosis, inflammation, DNA damage, and carcinogenesis¹¹.

The majority of cancer deaths (60% to 90%) are caused by metastases, which is the spread of cancer throughout the body⁵²⁶. Inflammation in the tumor microenvironment can promote and trigger the escape of metastatic tumor latency^{187,527}. NDMA-stimulated chronic inflammation can similarly ignite the escape of metastatic tumor latency and dormancy escape.

Angiogenesis (the growth of new blood vessels) can promote tumor growth via inflammation triggered by carcinogens¹¹. Carcinogens promote cancer via one or more of the 10 key characteristics including genotoxic and non-genotoxic mechanisms¹³. My laboratory recently demonstrated that carcinogen-induced cell death can trigger tumor dormancy escape by promoting inflammation and triggering oxidative stress (e.g., endoplasmic reticulum (ER) stress response)¹¹. Oxidative stress can, in addition to inducing the formation of mutations in DNA molecules, promote survival and proliferation of cancer cells with DNA mutations.

Our laboratory showed that carcinogen-generated cell death stimulated inflammation via pro-inflammatory molecules called cytokines and lipids as well as oxidative stress genes¹¹. Cancer can result from excessive cell death and non-resolving inflammation in the tumor microenvironment^{529,530}. Thus, chronic inflammation and pro-inflammatory molecules cause oxidative stress (key characteristic #5) and act synergistically with DNA damage to drive mutations and carcinogenesis^{171,177,531,532}.

Since NDMA stimulates inflammation (key characteristic #6), immunosuppression (key characteristic #7) and other tumor-promoting processes such as oxidative stress, NDMA can trigger tumor dormancy escape and reduce tumor latency. **Thus, it is highly likely that many patients exposed to NDMA and/or NDEA from valsartan already had dormant tumors that were then triggered via exposure to these carcinogens.**

NDMA and NDEA are synergistic in causing cancer

Synergistic refers to the combination of chemicals to create a combined effect greater than the sum of their separate effects. This is what happens when NDMA and NDEA are combined. Some of the contaminated valsartan tablets contained both NDMA and NDEA. As these two potent

carcinogens work synergistically, the combined toxic effect of both being in one tablet would be greater than the sum of their individual effect.

One study was designed to investigate the potential synergistic cancer-causing activity of more than one nitrosamine and add the carcinogenic effects of very low dose nitrosamines including NDEA. In this study, the three nitroso compounds chosen had been individually tested in dose—response studies. Based on these experiments it was possible to estimate doses which would induce < 50% tumors in their target organ when administered throughout the life of the animal²²⁵.

The three nitrosamines included NDEA. In this study, up to 45% of animals treated with NDEA developed liver (e.g., hepatocellular tumors). The gastrointestinal tract ranked second among the organs affected by NDEA: 31, 9 and 11 % of all rats were found to bear tumors in the digestive tract compared to 5% in the control group. Tumors in the oral cavity and in the esophagus accounted for the increase in tumor incidence in the gastrointestinal along with kidney tumors in the NDEA-treated groups. Combined administration of NDEA, NPYR and NDE1A, i.e., the highest combination dosage, was associated with a 1.7-fold significant increase in malignant tumors included tumors of the hematopoietic and lymphatic system. It was demonstrated in rats that the hepatotropic carcinogenicity of these genotoxic N-nitrosamines is additive when they are administered in combination²²⁵. Besides the liver, other organs revealed a significant increase in tumor incidence only when at least two of the three compounds administered in combination effected a treatment- related increase in tumor incidences (gastrointestinal tract, neurogenic tissue, urinary tract) over individual administrations. Notably, of the nitrosamines tested, NDEA was found to be the most carcinogenic agent as it contributed by over 17 orders of magnitude more to

the relative risk of dying with liver tumor. Adding two or more nitrosamines was found to be additive or synergistic in causing cancer^{372,374}.

At low concentrations, a chemical mixture has synergistic pro-tumorigenic activity on benign and malignant cells at a significantly lower concentrations than single chemicals⁵³³. The combination of the carcinogens NDMA and NDEA was more genotoxic (e.g., increased DNA double-strand breaks), carcinogenic, and mutagenic compared to single exposure and the mixture of carcinogens was highly mutagenic, genotoxic, and carcinogenic¹³⁷. *Thus, Valsartan contaminated with both NDMA and NDEA would likely be more carcinogenic in humans than exposure to either carcinogen alone.* There is increased mutagenesis, genotoxicity, and carcinogenic effects by NDMA and NDEA. The combination of nitrosamines (e.g. NDMA and NDEA) transforms normal (non-cancer) NIH3T3 cells called fibroblasts into cancer cells.²⁹⁹.

Future Risk of Recurrence or Spread

Patients who develop cancers from contaminated valsartan are at risk for cancer spread and recurrence once their cancer is removed by surgery and/or treated with chemotherapy and radiation. Cancer may recur after removal at any time and most cancer patients die from cancer recurrence or spread (called metastasis) of their cancer. Thus, cancer patients exposed to contaminated valsartan will require life-long surveillance to monitor for cancer recurrence and spread of their cancer.

There can be a tumor dormancy state with slow growing disease in patients who have their cancer surgically removed. Cancer studies show that malignant cells that disseminate early can

reside as single cells or as micro metastatic clusters in the bone marrow^{487,488}. These disseminated tumor cells lack the ability to colonize or are prevented from colonizing by the environment, resulting in a state of proliferative dormancy⁴⁸⁹.

A clear strategy to detect cancer after cancer surgery is challenging and not always possible. Thus, close lifelong follow-up is recommended for cancer patients exposed to valsartan contaminated NDMA. Many cancer patients do not die from their primary tumor but from metastasis or recurrent tumors⁴⁹⁰.

Over the past century, the cancer field has evolved from a “more is better” approach with increasingly radical surgeries to a view of early-stage cancer as a systemic disease, resulting in the adoption of chemotherapy⁹. However, my laboratory and others have demonstrated that cancer treatment is a double-edged sword, as surgery including biopsy, chemotherapy, or radiation can induce tumor-dormancy escape and reduce tumor latency via inflammation and immunosuppression^{6,7,9,500,501,534-543}. Even anesthesia during surgery can impair the resolution of inflammation⁵⁴⁴. My laboratory has demonstrated chemotherapy-generated cell death can paradoxically promote tumor growth via the release of proinflammatory and proangiogenic molecules including cytokines and lipids (called eicosanoids)^{6,7,10}. Cancer surgery can stimulate the metastatic process by inducing tumor dormancy escape of micro metastatic lesions^{9,495,545,546}. Local cancer recurrence (when a cancer returns after cancer treatment) following surgery to remove the cancer provides support for tumor dormancy^{495,545,546}. In landmark studies from the University of Pittsburgh and MIT, surgery was demonstrated to promote metastasis and dormancy escape, not only from mechanical dissemination of cancer cells, but also by stimulation of chronic inflammation (key characteristic #6) and surgery-associated immunosuppression, resulting in the growth of dormant cancer cells throughout the body^{500,501}.

Although chemotherapy increases overall survival in select cancer patients, it may also harbor inherent tumor-promoting activity limiting its effective anti-tumor activity^{6,7,10,547-549}. While chemotherapy remains a front-line treatment for many cancers, various animal models suggest that chemotherapy may stimulate or induce tumor initiation, growth, and metastasis⁵⁴⁸⁻⁵⁵⁷.

For decades, cancer therapy has focused on killing cancer cells to reduce tumor burden. However, in 1956, Révész demonstrated that co-injection of irradiated dead cells with tumor cells dramatically reduced the number of tumor cells needed to cause cancer in rodents⁵⁴¹, which has been confirmed in multiple tumor models. Thus, cancer therapy may inherently be a double-edged sword as radiation-induced dead tumor cells can promote tumor growth (the Révész Phenomenon)^{541,558-561}. Thus, cell death can paradoxically promote tumor growth by stimulating the living tumor cells via inflammation^{6,558}. High levels of cell death in tumors of patients with cancer have also been shown to correlate with poor prognosis in several cancer types including colorectal, bladder, breast, head, neck and lung and may be causatively involved in tumor growth⁵⁶²⁻⁵⁷⁰. Chemotherapy and radiation also activate the cell death marker caspase-3, which is associated with poor patient outcome after chemotherapy^{571,572}. Carcinogen-induced mutations of the DNA in normal cells leads to apoptotic cell death that creates a protumorigenic microenvironment consisting of proinflammatory mediators¹¹.

Cancer surgery itself places patients at risk for tumor recurrence, as surgery in the setting of cancer increases the chance of tumor recurrence and cancer spread^{345,363-366}. Surgery alone stimulates tumor dormancy escape^{345,366}. Surgery can stimulate pro-inflammatory and pro-angiogenic factors that can stimulate dormant tumor cells to escape and form a growing tumor years after the initial primary tumor is removed via surgery^{363,367-370}. In an adult model of chronic wounding in zebrafish, wounding with subsequent inflammation leads to a greater incidence of

cancers³⁷¹. Surgery creates oxidative stress which can increase the chance of a tumor development³⁷⁰. Surgery itself can stimulate tumor growth via inflammation and angiogenesis³⁷². Because NDMA stimulates inflammation and surgery can stimulate tumor dormancy escape, patients' exposure to NDMA or NDEA who have surgery can put them at risk for developing primary and secondary cancers.

For similar reasons, continued exposure to NDMA and/or NDEA can cause an existing cancer to grow, metastasize and otherwise interfere with cancer therapy. NDMA and NDEA can both act as tumor promoters via the nine key characteristics such as inflammation, angiogenesis and oxidative stress which are known to cause tumors to grow and metastasize.

There is an Increased Risk of a Second Tumor Once Diagnosed with a Primary (First) Tumor

One of the consequences of being diagnosed with cancer is the increased risk of being diagnosed with a second primary cancer⁵⁷³. This is different from a recurrence or metastasis from the original primary (first) tumor. A second cancer can still be related to exposure to a carcinogen causing the primary (first) tumor^{574,575}. Patients diagnosed with cancer have a permanent increased risk of developing secondary or even more tumors, defined as "tumor(s) distinct from the original tumor"⁵⁷⁶⁻⁵⁷⁹. Cancer survivors always remain at an increased risk of subsequent malignancies⁵⁸⁰.

A total of 23,580 secondary invasive primary cancers were observed in follow-up among 204,962 cancer patients. Both males and females within the study cohort were found to have a significant excess risk of developing a second cancer relative to the incidence of cancer in the general population⁵⁷³. Cancer surgery itself places valsartan-contaminated cancer patients at risk for tumor recurrence, as surgery in the setting of cancer increases the chance of tumor recurrence and cancer spread^{501,535,540,581,582} and stimulates tumor dormancy escape^{501,535}. Surgery can stimulate pro-inflammatory and pro-angiogenic factors that can stimulate dormant tumor cells to

escape and form a growing tumor years after the initial primary tumor is removed via surgery^{540,583-586}.

CONCLUSION

As set forth in my Summary of Expert Opinions based upon the evidence in total, it is my opinion with a reasonable degree of medical and scientific certainty that the exposure to NDMA and/or NDEA in valsartan containing drugs increases the risk of, has caused and can cause future human cancer.

References

- 1 Folkman, J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* **6**, 273-286, doi:nrd2115 [pii] 10.1038/nrd2115 (2007).
- 2 Panigrahy, D. *et al.* PPARgamma ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *J Clin Invest* **110**, 923-932, doi:10.1172/JCI15634 (2002).
- 3 Kaipainen, A. *et al.* PPARalpha deficiency in inflammatory cells suppresses tumor growth. *PLoS One* **2**, e260, doi:10.1371/journal.pone.0000260 (2007).
- 4 Panigrahy, D. *et al.* Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J Clin Invest* **122**, 178-191, doi:10.1172/JCI58128 58128 [pii] (2012).
- 5 Panigrahy, D. *et al.* Epoxyeicosanoids promote organ and tissue regeneration. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1311565110 (2013).
- 6 Sulciner, M. L. *et al.* Resolvins suppress tumor growth and enhance cancer therapy. *J Exp Med* **215**, 115-140, doi:10.1084/jem.20170681 (2018).
- 7 Gartung, A. *et al.* Suppression of chemotherapy-induced cytokine/lipid mediator surge and ovarian cancer by a dual COX-2/sEH inhibitor. *Proc Natl Acad Sci U S A* **116**, 1698-1703, doi:10.1073/pnas.1803999116 (2019).

- 8 Gilligan, M. M. *et al.* Aspirin-triggered proresolving mediators stimulate resolution in cancer. *Proc Natl Acad Sci U S A* **116**, 6292-6297, doi:10.1073/pnas.1804000116 (2019).
- 9 Panigrahy, D. *et al.* Preoperative stimulation of resolution and inflammation blockade eradicates micrometastases. *J Clin Invest* **129**, 2964-2979, doi:10.1172/JCI127282 (2019).
- 10 Chang, J. *et al.* Chemotherapy-generated cell debris stimulates colon carcinoma tumor growth via osteopontin. *FASEB J* **33**, 114-125, doi:10.1096/fj.201800019RR (2019).
- 11 Fishbein, A. *et al.* Resolution of eicosanoid/cytokine storm prevents carcinogen and inflammation-initiated hepatocellular cancer progression. *Proc Natl Acad Sci U S A* **117**, 21576-21587, doi:10.1073/pnas.2007412117 (2020).
- 12 Panigrahy, D. *et al.* Inflammation resolution: a dual-pronged approach to averting cytokine storms in COVID-19? *Cancer Metastasis Rev* **39**, 337-340, doi:10.1007/s10555-020-09889-4 (2020).
- 13 Fishbein, A., Hammock, B. D., Serhan, C. N. & Panigrahy, D. Carcinogenesis: Failure of resolution of inflammation? *Pharmacol Ther* **218**, 107670, doi:10.1016/j.pharmthera.2020.107670 (2021).
- 14 Panigrahy, D., Gilligan, M. M., Serhan, C. N. & Kashfi, K. Resolution of inflammation: An organizing principle in biology and medicine. *Pharmacol Ther*, 107879, doi:10.1016/j.pharmthera.2021.107879 (2021).
- 15 Sulciner, M. L., Gartung, A., Gilligan, M. M., Serhan, C. N. & Panigrahy, D. Targeting lipid mediators in cancer biology. *Cancer Metastasis Rev* **37**, 557-572, doi:10.1007/s10555-018-9754-9 (2018).
- 16 Chiolerio, A. & Burnier, M. Pharmacology of valsartan, an angiotensin II receptor antagonist. *Expert Opin Investig Drugs* **7**, 1915-1925, doi:10.1517/13543784.7.11.1915 (1998).
- 17 Ibrahim, H. K. & El-Setouhy, D. A. Valsartan orodispersible tablets: formulation, in vitro/in vivo characterization. *AAPS PharmSciTech* **11**, 189-196, doi:10.1208/s12249-009-9354-7 (2010).
- 18 Charoo, N. A., Ali, A. A., Buha, S. K. & Rahman, Z. Lesson Learnt from Recall of Valsartan and Other Angiotensin II Receptor Blocker Drugs Containing NDMA and NDEA Impurities. *AAPS PharmSciTech* **20**, 166, doi:10.1208/s12249-019-1376-1 (2019).
- 19 Peto, R., Gray, R., Brantom, P. & Grasso, P. Effects on 4080 rats of chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine: a detailed dose-response study. *Cancer Res* **51**, 6415-6451 (1991).
- 20 Miller, F. J., Schlosser, P. M. & Janszen, D. B. Haber's rule: a special case in a family of curves relating concentration and duration of exposure to a fixed level of response for a given endpoint. *Toxicology* **149**, 21-34, doi:10.1016/s0300-483x(00)00229-8 (2000).
- 21 Luch, A. Nature and nurture - lessons from chemical carcinogenesis. *Nat Rev Cancer* **5**, 113-125, doi:10.1038/nrc1546 (2005).
- 22 Pitot, H. C. The molecular biology of carcinogenesis. *Cancer* **72**, 962-970, doi:10.1002/1097-0142(19930801)72:3+<962::aid-cnrc2820721303>3.0.co;2-h (1993).
- 23 Mulware, S. J. Trace elements and carcinogenicity: a subject in review. *3 Biotech* **3**, 85-96, doi:10.1007/s13205-012-0072-6 (2013).
- 24 Smith, M. T. *et al.* Key Characteristics of Carcinogens as a Basis for Organizing Data on Mechanisms of Carcinogenesis. *Environ Health Perspect* **124**, 713-721, doi:10.1289/ehp.1509912 (2016).
- 25 Smith, M. T. *et al.* The Key Characteristics of Carcinogens: Relationship to the Hallmarks of Cancer, Relevant Biomarkers, and Assays to Measure Them. *Cancer Epidemiol Biomarkers Prev*, doi:10.1158/1055-9965.EPI-19-1346 (2020).
- 26 Hecht, S. S. & Szabo, E. Fifty years of tobacco carcinogenesis research: from mechanisms to early detection and prevention of lung cancer. *Cancer Prev Res (Phila)* **7**, 1-8, doi:10.1158/1940-6207.CAPR-13-0371 (2014).

- 27 Hecht, S. S. Research opportunities related to establishing standards for tobacco products under the Family Smoking Prevention and Tobacco Control Act. *Nicotine Tob Res* **14**, 18-28, doi:10.1093/ntr/ntq216 (2012).
- 28 Maronpot, R. R., Flake, G. & Huff, J. Relevance of animal carcinogenesis findings to human cancer predictions and prevention. *Toxicol Pathol* **32 Suppl 1**, 40-48 (2004).
- 29 Calabrese, E. J. & Blain, R. B. The Single Exposure Carcinogen Database: assessing the circumstances under which a single exposure to a carcinogen can cause cancer. *Toxicol Sci* **50**, 169-185, doi:10.1093/toxsci/50.2.169 (1999).
- 30 Nohmi, T. Thresholds of Genotoxic and Non-Genotoxic Carcinogens. *Toxicol Res* **34**, 281-290, doi:10.5487/TR.2018.34.4.281 (2018).
- 31 Nohmi, T. & Matsumoto, K. Effects of DNA polymerase kappa and mismatch repair on dose-responses of chromosome aberrations induced by three oxidative genotoxins in human cells. *Environ Mol Mutagen* **61**, 193-199, doi:10.1002/em.22315 (2020).
- 32 Tomatis, L. *et al.* Avoided and avoidable risks of cancer. *Carcinogenesis* **18**, 97-105 (1997).
- 33 Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. *Nature* **454**, 436-444, doi:10.1038/nature07205 (2008).
- 34 Krewski, D. *et al.* Key characteristics of 86 agents known to cause cancer in humans. *J Toxicol Environ Health B Crit Rev* **22**, 244-263, doi:10.1080/10937404.2019.1643536 (2019).
- 35 Fung, V. A., Barrett, J. C. & Huff, J. The carcinogenesis bioassay in perspective: application in identifying human cancer hazards. *Environ Health Perspect* **103**, 680-683, doi:10.1289/ehp.95103680 (1995).
- 36 Tomatis, L., Melnick, R. L., Haseman, J., Barrett, J. C. & Huff, J. Alleged misconceptions' distort perceptions of environmental cancer risks. *FASEB J* **15**, 195-203, doi:10.1096/fj.99-1056com (2001).
- 37 Cogliano, V. J. Current criteria to establish human carcinogens. *Semin Cancer Biol* **14**, 407-412, doi:10.1016/j.semcancer.2004.06.003 (2004).
- 38 Cogliano, V. J. *et al.* The science and practice of carcinogen identification and evaluation. *Environ Health Perspect* **112**, 1269-1274 (2004).
- 39 Hill, A. B. The Environment and Disease: Association or Causation? *Proc R Soc Med* **58**, 295-300 (1965).
- 40 Huff, J., Haseman, J. & Rall, D. Scientific concepts, value, and significance of chemical carcinogenesis studies. *Annu Rev Pharmacol Toxicol* **31**, 621-652, doi:10.1146/annurev.pa.31.040191.003201 (1991).
- 41 Tomatis, L., Aitio, A., Wilbourn, J. & Shuker, L. Human carcinogens so far identified. *Jpn J Cancer Res* **80**, 795-807 (1989).
- 42 Tomatis, L. The predictive value of rodent carcinogenicity tests in the evaluation of human risks. *Annu Rev Pharmacol Toxicol* **19**, 511-530, doi:10.1146/annurev.pa.19.040179.002455 (1979).
- 43 Huff, J. Chemicals studied and evaluated in long-term carcinogenesis bioassays by both the Ramazzini Foundation and the National Toxicology Program: in tribute to Cesare Maltoni and David Rall. *Ann N Y Acad Sci* **982**, 208-230, doi:10.1111/j.1749-6632.2002.tb04935.x (2002).
- 44 Harris, C. C. *et al.* Metabolism of dimethylnitrosamine and 1,2-dimethylhydrazine in cultured human bronchi. *Cancer Res* **37**, 2309-2311 (1977).
- 45 Chung, S. K., Lee, A. Y. & Chung, S. S. Mouse models for human diseases. *Hong Kong Med J* **3**, 201-209 (1997).
- 46 Bertram, J. S., Kolonel, L. N. & Meyskens, F. L., Jr. Rationale and strategies for chemoprevention of cancer in humans. *Cancer Res* **47**, 3012-3031 (1987).
- 47 Boyd, J. A. & Barrett, J. C. Genetic and cellular basis of multistep carcinogenesis. *Pharmacol Ther* **46**, 469-486, doi:10.1016/0163-7258(90)90028-z (1990).

- 48 Wilbourn, J. *et al.* Response of experimental animals to human carcinogens: an analysis based upon the IARC Monographs programme. *Carcinogenesis* **7**, 1853-1863 (1986).
- 49 Ziegler, A., Gonzalez, L. & Blikslager, A. Large Animal Models: The Key to Translational Discovery in Digestive Disease Research. *Cell Mol Gastroenterol Hepatol* **2**, 716-724, doi:10.1016/j.jcmgh.2016.09.003 (2016).
- 50 Kelly, M. G. *et al.* Induction of hepatic cell carcinomas in monkeys with N-nitrosodiethylamine. *J Natl Cancer Inst* **36**, 323-351 (1966).
- 51 Swindle, M. M., Makin, A., Herron, A. J., Clubb, F. J., Jr. & Frazier, K. S. Swine as models in biomedical research and toxicology testing. *Vet Pathol* **49**, 344-356, doi:10.1177/0300985811402846 (2012).
- 52 Huff, J. Chemicals and cancer in humans: first evidence in experimental animals. *Environ Health Perspect* **100**, 201-210, doi:10.1289/ehp.93100201 (1993).
- 53 Tomatis, L. Prenatal exposure to chemical carcinogens and its effect on subsequent generations. *Natl Cancer Inst Monogr*, 159-184 (1979).
- 54 Cogliano, V. J. *et al.* Use of mechanistic data in IARC evaluations. *Environ Mol Mutagen* **49**, 100-109, doi:10.1002/em.20370 (2008).
- 55 Guyton, K. Z. *et al.* Application of the key characteristics of carcinogens in cancer hazard identification. *Carcinogenesis* **39**, 614-622, doi:10.1093/carcin/bgy031 (2018).
- 56 Hong, J. Y. *et al.* Metabolism of carcinogenic nitrosamines by rat nasal mucosa and the effect of diallyl sulfide. *Cancer Res* **51**, 1509-1514 (1991).
- 57 Ratajczak-Wrona, W., Jablonska, E., Garley, M., Jablonski, J. & Radziwon, P. Role of ERK1/2 kinase in the expression of iNOS by NDMA in human neutrophils. *Indian J Exp Biol* **51**, 73-80 (2013).
- 58 Sharma, V. & Singh, M. Ameliorative Effects of Operculina turpethum and its Isolated Stigma-5,22dien-3-o-beta-D-glucopyranoside on the Hematological Parameters of Male Mice Exposed to N-Nitrosodimethylamine, a Potent Carcinogen. *Toxicol Int* **21**, 29-36, doi:10.4103/0971-6580.128789 (2014).
- 59 Zhao, Y. Y. *et al.* Formation of N-nitrosamines from eleven disinfection treatments of seven different surface waters. *Environ Sci Technol* **42**, 4857-4862 (2008).
- 60 Zhou, W. J., Boyd, J. M., Qin, F., Hrudey, S. E. & Li, X. F. Formation of N-nitrosodiphenylamine and two new N-containing disinfection byproducts from chloramination of water containing diphenylamine. *Environ Sci Technol* **43**, 8443-8448, doi:10.1021/es901935v (2009).
- 61 Chen, W. H. & Young, T. M. Influence of nitrogen source on NDMA formation during chlorination of diuron. *Water Res* **43**, 3047-3056, doi:10.1016/j.watres.2009.04.020 (2009).
- 62 Yin, Y. *et al.* Probabilistic health risk assessment of nitrosamines in drinking water of Shaoxing, Zhejiang, China. *Environ Sci Pollut Res Int*, doi:10.1007/s11356-018-4026-3 (2019).
- 63 Swenberg, J. A., Hoel, D. G. & Magee, P. N. Mechanistic and statistical insight into the large carcinogenesis bioassays on N-nitrosodiethylamine and N-nitrosodimethylamine. *Cancer Res* **51**, 6409-6414 (1991).
- 64 Peto, R., Gray, R., Brantom, P. & Grasso, P. Nitrosamine carcinogenesis in 5120 rodents: chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age of starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters). *IARC Sci Publ*, 627-665 (1984).
- 65 Zhang, O. *et al.* Experimental and Theoretical Investigation of Effects of Ethanol and Acetic Acid on Carcinogenic NDMA Formation in Simulated Gastric Fluid. *J Phys Chem A* **120**, 4505-4513, doi:10.1021/acs.jpca.6b02582 (2016).

- 66 Dennehy, M. K. & Loeppky, R. N. Mass spectrometric methodology for the determination of glyoxaldehydoxyguanosine and O6-hydroxyethyldeoxyguanosine DNA adducts produced by nitrosamine bident carcinogens. *Chem Res Toxicol* **18**, 556-565, doi:10.1021/tx049802o (2005).
- 67 Fitzgerald, D. J. & Robinson, N. I. Development of a tolerable daily intake for N-nitrosodimethylamine using a modified benchmark dose methodology. *J Toxicol Environ Health A* **70**, 1670-1678, doi:10.1080/15287390701434844 (2007).
- 68 Hrudey, S. E., Bull, R. J., Cotruvo, J. A., Paoli, G. & Wilson, M. Drinking water as a proportion of total human exposure to volatile N-nitrosamines. *Risk Anal* **33**, 2179-2208, doi:10.1111/risa.12070 (2013).
- 69 Zhou, Q. *et al.* Field evidence of biodegradation of N-Nitrosodimethylamine (NDMA) in groundwater with incidental and active recycled water recharge. *Water Res* **43**, 793-805, doi:10.1016/j.watres.2008.11.011 (2009).
- 70 Preussmann, R. Occurrence and exposure to N-nitroso compounds and precursors. *IARC Sci Publ*, 3-15 (1984).
- 71 Yamaguchi, M., Takai, S., Hosono, A. & Seki, T. Bovine milk-derived alpha-lactalbumin inhibits colon inflammation and carcinogenesis in azoxymethane and dextran sodium sulfate-treated mice. *Biosci Biotechnol Biochem* **78**, 672-679, doi:10.1080/09168451.2014.890034 (2014).
- 72 Hattori, N. *et al.* Antibiotics suppress colon tumorigenesis through inhibition of aberrant DNA methylation in an azoxymethane and dextran sulfate sodium colitis model. *Cancer Sci* **110**, 147-156, doi:10.1111/cas.13880 (2019).
- 73 Yang, H. *et al.* A common antimicrobial additive increases colonic inflammation and colitis-associated colon tumorigenesis in mice. *Sci Transl Med* **10**, doi:10.1126/scitranslmed.aan4116 (2018).
- 74 Peto, R., Gray, R., Brantom, P. & Grasso, P. Dose and time relationships for tumor induction in the liver and esophagus of 4080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. *Cancer Res* **51**, 6452-6469 (1991).
- 75 Terracini, B. & Magee, P. N. Renal Tumours in Rats Following Injection of Dimethylnitrosamine at Birth. *Nature* **202**, 502-503, doi:10.1038/202502a0 (1964).
- 76 Khudoley, V. V. The induction of tumors in *Rana temporaria* with nitrosamines. *Neoplasma* **24**, 249-251 (1977).
- 77 Arai, M. *et al.* Long-term experiment of maximal non-carcinogenic dose of dimethylnitrosamine for carcinogenesis in rats. *Gan* **70**, 549-558 (1979).
- 78 Moiseev, G. E. & Benemanskii, V. V. [The carcinogenic activity of small concentrations of nitrosodimethylamine when inhaled]. *Vopr Onkol* **21**, 107-109 (1975).
- 79 Klein, R. G. *et al.* Effects of long-term inhalation of N-nitrosodimethylamine in rats. *IARC Sci Publ*, 322-328 (1991).
- 80 Klein, R. G. *et al.* Effect of long-term inhalation of N-nitroso-dimethylamine (NDMA) and SO₂/NO_x in rats. *Exp Pathol* **37**, 273-280 (1989).
- 81 Otsuka, H. & Kuwahara, A. Hemangiomatic lesions of mice treated with nitrosodimethylamine. *Gan* **62**, 147-156 (1971).
- 82 Kuwahara, A., Otsuka, H. & Nagamatsu, A. Induction of hemangiomatic lesions with dimethylnitrosoamine: influence of route of administration and strain of mice. *Gan* **63**, 499-502 (1972).
- 83 Terracini, B., Palestro, G., Gigliardi, M. R., Montesano, G. & Montesano, R. Carcinogenicity of dimethylnitrosamine in Swiss mice. *Br J Cancer* **20**, 871-876, doi:10.1038/bjc.1966.100 (1966).
- 84 Terracini, B., Magee, P. N. & Barnes, J. M. Hepatic pathology in rats on low dietary levels of dimethylnitrosamine. *Br J Cancer* **21**, 559-565 (1967).

- 85 Homburger, F. *et al.* Adenocarcinoma of the glandular stomach following 3-methylcholanthrene, N-nitrosodiethylamine, or N-nitrosodimethylamine feeding in carcinogen-susceptible inbred Syrian. *J Natl Cancer Inst* **57**, 141-144, doi:10.1093/jnci/57.1.141 (1976).
- 86 Tomatis, L. & Cefis, F. The effects of multiple and single administration of dimethylnitrosamine to hamsters. *Tumori* **53**, 447-451 (1967).
- 87 Campbell, J. S., Wiberg, G. S., Grice, H. C. & Lou, P. Stromal nephromas and renal cell tumors in suckling and weaned rats. *Cancer Res* **34**, 2399-2404 (1974).
- 88 Preussmann, R. Dose-response studies and 'no-effect-levels' of N-nitroso compounds: some general aspects. *Oncology* **37**, 243-250, doi:10.1159/000225445 (1980).
- 89 Magee, P. N. & Barnes, J. M. The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *Br J Cancer* **10**, 114-122 (1956).
- 90 Magee, P. N. & Barnes, J. M. Induction of kidney tumours in the rat with dimethylnitrosamine (N-nitrosodimethylamine). *J Pathol Bacteriol* **84**, 19-31 (1962).
- 91 Le Page, R. N. & Christie, G. S. Induction of liver tumours in the rabbit by feeding dimethylnitrosamine. *Br J Cancer* **23**, 125-131, doi:10.1038/bjc.1969.18 (1969).
- 92 Le Page, R. N. & Christie, G. S. Induction of liver tumours in the guinea pig by feeding dimethylnitrosamine. *Pathology* **1**, 49-56, doi:10.3109/00313026909061034 (1969).
- 93 Lijinsky, W. & Reuber, M. D. Carcinogenesis in rats by nitrosodimethylamine and other nitrosomethylalkylamines at low doses. *Cancer Lett* **22**, 83-88, doi:10.1016/0304-3835(84)90047-8 (1984).
- 94 Lijinsky, W. & Reuber, M. D. Comparative carcinogenesis by some aliphatic nitrosamines in Fischer rats. *Cancer Lett* **14**, 297-302, doi:10.1016/0304-3835(81)90158-0 (1981).
- 95 Lijinsky, W., Kovatch, R. M. & Riggs, C. W. Carcinogenesis by nitrosodialkylamines and azoxyalkanes given by gavage to rats and hamsters. *Cancer Res* **47**, 3968-3972 (1987).
- 96 Clapp, N. K., Craig, A. W. & Toya, R. E., Sr. Pulmonary and hepatic oncogenesis during treatment of male RF mice with dimethyl-nitrosamine. *J Natl Cancer Inst* **41**, 1213-1227 (1968).
- 97 Clapp, N. K. & Toya, R. E. Effect of cumulative dose and dose rate on dimethylnitrosamine oncogenesis in RF mice. *J Natl Cancer Inst* **45**, 495-498 (1970).
- 98 Clapp, N. K., Tyndall, R. L. & Otten, J. A. Differences in tumor types and organ susceptibility in BALB-c and RF mice following dimethylnitrosamine and diethylnitrosamine. *Cancer Res* **31**, 196-198 (1971).
- 99 Druckrey, H., Preussmann, R., Ivankovic, S. & Schmahl, D. [Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats]. *Z Krebsforsch* **69**, 103-201 (1967).
- 100 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
- 101 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:S0092-8674(11)00127-9 [pii] 10.1016/j.cell.2011.02.013 (2011).
- 102 Nicoll, J. W., Swann, P. F. & Pegg, A. E. Effect of dimethylnitrosamine on persistence of methylated guanines in rat liver and kidney DNA. *Nature* **254**, 261-262, doi:10.1038/254261a0 (1975).
- 103 Hecht, S. S. Lung carcinogenesis by tobacco smoke. *Int J Cancer* **131**, 2724-2732, doi:10.1002/ijc.27816 (2012).
- 104 Miller, J. A. Carcinogenesis by chemicals: an overview--G. H. A. Clowes memorial lecture. *Cancer Res* **30**, 559-576 (1970).
- 105 Kang, J. S., Wanibuchi, H., Morimura, K., Gonzalez, F. J. & Fukushima, S. Role of CYP2E1 in diethylnitrosamine-induced hepatocarcinogenesis in vivo. *Cancer Res* **67**, 11141-11146, doi:10.1158/0008-5472.CAN-07-1369 (2007).

- 106 Nishimura, M., Yaguti, H., Yoshitsugu, H., Naito, S. & Satoh, T. Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi* **123**, 369-375, doi:10.1248/yakushi.123.369 (2003).
- 107 Archer, M. C. Mechanisms of action of N-nitroso compounds. *Cancer Surv* **8**, 241-250 (1989).
- 108 Souliotis, V. L., Chhabra, S., Anderson, L. M. & Kyrtopoulos, S. A. Dosimetry of O6-methylguanine in rat DNA after low-dose, chronic exposure to N-nitrosodimethylamine (NDMA). Implications for the mechanism of NDMA hepatocarcinogenesis. *Carcinogenesis* **16**, 2381-2387, doi:10.1093/carcin/16.10.2381 (1995).
- 109 Lee, V. M., Cameron, R. G. & Archer, M. C. The role of hepatocyte heterogeneity in the initiation of hepatocarcinogenesis. *Carcinogenesis* **14**, 1403-1408 (1993).
- 110 Lee, V. M., Keefer, L. K. & Archer, M. C. An evaluation of the roles of metabolic denitrosation and alpha-hydroxylation in the hepatotoxicity of N-Nitrosodimethylamine. *Chem Res Toxicol* **9**, 1319-1324, doi:10.1021/tx960077u (1996).
- 111 Encell, L., Foiles, P. G. & Gold, B. The relationship between N-nitrosodimethylamine metabolism and DNA methylation in isolated rat hepatocytes. *Carcinogenesis* **17**, 1127-1134, doi:10.1093/carcin/17.5.1127 (1996).
- 112 Czygan, P. *et al.* Microsomal metabolism of dimethylnitrosamine and the cytochrome P-450 dependency of its activation to a mutagen. *Cancer Res* **33**, 2983-2986 (1973).
- 113 Argus, M. F., Arcos, J. C., Pastor, K. M., Wu, B. C. & Venkatesan, N. Dimethylnitrosamine-demethylase: absence of increased enzyme catabolism and multiplicity of effector sites in repression. Hemoprotein involvement. *Chem Biol Interact* **13**, 127-140, doi:10.1016/0009-2797(76)90002-8 (1976).
- 114 Lawley, P. D. Some chemical aspects of dose-response relationships in alkylation mutagenesis. *Mutat Res* **23**, 283-295, doi:10.1016/0027-5107(74)90102-x (1974).
- 115 O'Connor, P. J., Capps, M. J. & Craig, A. W. Comparative studies of the hepatocarcinogen N,N-dimethylnitrosamine in vivo: reaction sites in rat liver DNA and the significance of their relative stabilities. *Br J Cancer* **27**, 153-166, doi:10.1038/bjc.1973.19 (1973).
- 116 Pegg, A. E. Formation and metabolism of alkylated nucleosides: possible role in carcinogenesis by nitroso compounds and alkylating agents. *Adv Cancer Res* **25**, 195-269, doi:10.1016/s0065-230x(08)60635-1 (1977).
- 117 Hard, G. C. & Butler, W. H. Cellular analysis of renal neoplasia: light microscope study of the development of interstitial lesions induced in the rat kidney by a single carcinogenic dose of dimethylnitrosamine. *Cancer Res* **30**, 2806-2815 (1970).
- 118 Devereux, T. R., Anderson, M. W. & Belinsky, S. A. Role of ras protooncogene activation in the formation of spontaneous and nitrosamine-induced lung tumors in the resistant C3H mouse. *Carcinogenesis* **12**, 299-303, doi:10.1093/carcin/12.2.299 (1991).
- 119 Fadlallah, S. *et al.* O6-methylguanine-DNA adducts in rat lymphocytes after in vivo exposure to N-nitrosodimethylamine (NDMA). *Int J Immunopharmacol* **16**, 583-591, doi:10.1016/0192-0561(94)90109-0 (1994).
- 120 Margison, G. P., Margison, J. M. & Montesano, R. Methylated purines in the deoxyribonucleic acid of various Syrian-golden-hamster tissues after administration of a hepatocarcinogenic dose of dimethylnitrosamine. *Biochem J* **157**, 627-634, doi:10.1042/bj1570627 (1976).
- 121 Anderson, L. M. *et al.* N-nitrosodimethylamine-derived O(6)-methylguanine in DNA of monkey gastrointestinal and urogenital organs and enhancement by ethanol. *Int J Cancer* **66**, 130-134, doi:10.1002/(SICI)1097-0215(19960328)66:1<130::AID-IJC22>3.0.CO;2-G (1996).
- 122 Chimpanzee, S. & Analysis, C. Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* **437**, 69-87, doi:10.1038/nature04072 (2005).

- 123 Yan, G. *et al.* Genome sequencing and comparison of two nonhuman primate animal models, the cynomolgus and Chinese rhesus macaques. *Nat Biotechnol* **29**, 1019-1023, doi:10.1038/nbt.1992 (2011).
- 124 Annola, K. *et al.* Transplacental transfer of nitrosodimethylamine in perfused human placenta. *Placenta* **30**, 277-283, doi:10.1016/j.placenta.2008.12.012 (2009).
- 125 Montesano, R. & Magee, P. N. Metabolism of diethylnitrosamine by human liver slices in vitro. *Nature* **228**, 173-174, doi:10.1038/228173a0 (1970).
- 126 Herron, D. C. & Shank, R. C. Methylated purines in human liver DNA after probable dimethylnitrosamine poisoning. *Cancer Res* **40**, 3116-3117 (1980).
- 127 Bartsch, H. & Montesano, R. Relevance of nitrosamines to human cancer. *Carcinogenesis* **5**, 1381-1393, doi:10.1093/carcin/5.11.1381 (1984).
- 128 Autrup, H., Grafstrom, R. C., Christensen, B. & Kieler, J. Metabolism of chemical carcinogens by cultured human and rat bladder epithelial cells. *Carcinogenesis* **2**, 763-768, doi:10.1093/carcin/2.8.763 (1981).
- 129 Autrup, H., Harris, C. C. & Trump, B. F. Metabolism of acyclic and cyclic N-nitrosamines by cultured human colon. *Proc Soc Exp Biol Med* **159**, 111-115 (1978).
- 130 Harris, C. C. *et al.* Metabolism of benzo(a)pyrene, N-nitrosodimethylamine, and N-nitrosopyrrolidine and identification of the major carcinogen-DNA adducts formed in cultured human esophagus. *Cancer Res* **39**, 4401-4406 (1979).
- 131 Chhabra, S. K. *et al.* O6-methylguanine DNA adduct formation and modulation by ethanol in placenta and fetal tissues after exposure of pregnant patas monkeys to N-nitrosodimethylamine. *Cancer Res* **55**, 6017-6020 (1995).
- 132 Zaidi, S. N. *et al.* Normal human breast xenografts activate N-nitrosodimethylamine: identification of potential target cells for an environmental nitrosamine. *Br J Cancer* **66**, 79-83, doi:10.1038/bjc.1992.220 (1992).
- 133 Cimino, M. C. Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. *Environ Mol Mutagen* **47**, 362-390, doi:10.1002/em.20216 (2006).
- 134 Kossler, N. *et al.* Identification of specific mRNA signatures as fingerprints for carcinogenesis in mice induced by genotoxic and nongenotoxic hepatocarcinogens. *Toxicol Sci* **143**, 277-295, doi:10.1093/toxsci/kfu248 (2015).
- 135 Bates, A. D., Neuteboom, I. & Hofker, M. A micronucleus technique for detecting clastogenic effects of mutagens/carcinogens (DEN, DMN) in hepatocytes of rat liver in vivo. *Mutat Res* **74**, 11-20, doi:10.1016/0165-1161(80)90187-9 (1980).
- 136 Bates, A. D., Neuteboom, I., de Vogel, N. & den Engelse, L. The induction of chromosomal damage in rat hepatocytes and lymphocytes. I. Time-dependent changes of the clastogenic effects of diethylnitrosamine, dimethylnitrosamine and ethyl methanesulfonate. *Mutat Res* **107**, 131-151, doi:10.1016/0027-5107(83)90084-2 (1983).
- 137 Bates, A. D. *et al.* Persistence of preclastogenic damage in hepatocytes of rats exposed to ethylnitrosourea, diethylnitrosamine, dimethylnitrosamine and methyl methanesulphonate. Correlation with DNA O-alkylation. *Carcinogenesis* **7**, 1053-1058, doi:10.1093/carcin/7.7.1053 (1986).
- 138 Bauknecht, T., Vogel, W., Bayer, U. & Wild, D. Comparative in vivo mutagenicity testing by SCE and micronucleus induction in mouse bone marrow. *Hum Genet* **35**, 299-307, doi:10.1007/BF00446622 (1977).
- 139 Wild, D. Cytogenetic effects in the mouse of 17 chemical mutagens and carcinogens evaluated by the micronucleus test. *Mutat Res* **56**, 319-327, doi:10.1016/0027-5107(78)90200-2 (1978).

- 140 Neft, R. E. & Conner, M. K. Induction of sister chromatid exchange in multiple murine tissues in vivo by various methylating agents. *Teratog Carcinog Mutagen* **9**, 219-237, doi:10.1002/tcm.1770090405 (1989).
- 141 Mehta, R. *et al.* Micronucleus formation induced in rat liver and esophagus by nitrosamines. *Cancer Lett* **35**, 313-320, doi:10.1016/0304-3835(87)90133-9 (1987).
- 142 Robbiano, L., Mereto, E., Migliazzi Morando, A., Pastore, P. & Brambilla, G. An in vivo micronucleus assay for detecting the clastogenic effect in rat kidney cells. *Mutat Res* **390**, 51-57, doi:10.1016/s0165-1218(96)00165-6 (1997).
- 143 Cllet, I., Fournier, E., Melcion, C. & Cordier, A. In vivo micronucleus test using mouse hepatocytes. *Mutat Res* **216**, 321-326, doi:10.1016/0165-1161(89)90042-3 (1989).
- 144 Odagiri, Y., Adachi, S., Katayama, H. & Takemoto, K. Detection of the cytogenetic effect of inhaled aerosols by the micronucleus test. *Mutat Res* **170**, 79-83, doi:10.1016/0165-1218(86)90084-4 (1986).
- 145 Inui, N., Nishi, Y., Taketomi, M. & Mori, M. Transplacental action of sodium nitrite on embryonic cells of Syrian golden hamster. *Mutat Res* **66**, 149-158, doi:10.1016/0165-1218(79)90060-0 (1979).
- 146 Bolognesi, C., Rossi, L. & Santi, L. A new method to reveal the genotoxic effects of N-nitrosodimethylamine in pregnant mice. *Mutat Res* **207**, 57-62, doi:10.1016/0165-7992(88)90042-5 (1988).
- 147 Laishes, B. A., Koropatnick, D. J. & Stich, H. F. Organ-specific DNA damage induced in mice by the organotropic carcinogens 4-nitroquinoline 1-oxide and dimethylnitrosamine. *Proc Soc Exp Biol Med* **149**, 978-982, doi:10.3181/00379727-149-38938 (1975).
- 148 Petzold, G. L. & Swenberg, J. A. Detection of DNA damage induced in vivo following exposure of rats to carcinogens. *Cancer Res* **38**, 1589-1594 (1978).
- 149 Abanobi, S. E., Farber, E. & Sarma, D. S. Persistence of DNA damage during development of liver angiosarcoma in rats fed dimethylnitrosamine. *Cancer Res* **39**, 1592-1596 (1979).
- 150 Mirsalis, J. C. & Butterworth, B. E. Detection of unscheduled DNA synthesis in hepatocytes isolated from rats treated with genotoxic agents: an in vivo- in vitro assay for potential carcinogens and mutagens. *Carcinogenesis* **1**, 621-625, doi:10.1093/carcin/1.7.621 (1980).
- 151 Brambilla, G., Cavanna, M., Pino, A. & Robbiano, L. Quantitative correlation among DNA damaging potency of six N-nitroso compounds and their potency in inducing tumor growth in bacterial mutations. *Carcinogenesis* **2**, 425-429, doi:10.1093/carcin/2.5.425 (1981).
- 152 Brambilla, G., Carlo, P., Finollo, R. & Sciaba, L. Dose-response curves for liver DNA fragmentation induced in rats by sixteen N-nitroso compounds as measured by viscometric and alkaline elution analyses. *Cancer Res* **47**, 3485-3491 (1987).
- 153 Bermudez, E., Mirsalis, J. C. & Eales, H. C. Detection of DNA damage in primary cultures of rat hepatocytes following in vivo and in vitro exposure to genotoxic agents. *Environ Mutagen* **4**, 667-679, doi:10.1002/em.2860040606 (1982).
- 154 Cesarone, C. F., Bolognesi, C. & Santi, L. DNA repair synthesis in mice spermatids after treatment with N-methyl-N-nitroso-urea and N-N-dimethyl-nitrosamine: preliminary results. *Toxicology* **12**, 183-186, doi:10.1016/0300-483x(79)90045-3 (1979).
- 155 Barbin, A., Bereziat, J. C. & Bartsch, H. Evaluation of DNA damage by the alkaline elution technique in liver, kidneys and lungs of rats and hamsters treated with N-nitrosodialkylamines. *Carcinogenesis* **4**, 541-545, doi:10.1093/carcin/4.5.541 (1983).
- 156 Doolittle, D. J., Bermudez, E., Working, P. K. & Butterworth, B. E. Measurement of genotoxic activity in multiple tissues following inhalation exposure to dimethylnitrosamine. *Mutat Res* **141**, 123-127, doi:10.1016/0165-7992(84)90023-x (1984).

- 157 Kornbrust, D. & Dietz, D. Aroclor 1254 pretreatment effects on DNA repair in rat hepatocytes elicited by in vivo or in vitro exposure to various chemicals. *Environ Mutagen* **7**, 857-870, doi:10.1002/em.2860070607 (1985).
- 158 Pool, B. L., Brendler, S. Y., Liegibel, U. M., Tompa, A. & Schmezer, P. Employment of adult mammalian primary cells in toxicology: in vivo and in vitro genotoxic effects of environmentally significant N-nitrosodialkylamines in cells of the liver, lung, and kidney. *Environ Mol Mutagen* **15**, 24-35 (1990).
- 159 Brendler, S. Y., Tompa, A., Hutter, K. F., Preussmann, R. & Pool-Zobel, B. L. In vivo and in vitro genotoxicity of several N-nitrosamines in extrahepatic tissues of the rat. *Carcinogenesis* **13**, 2435-2441 (1992).
- 160 Jorquera, R., Castonguay, A. & Schuller, H. M. Effects of age and ethanol on DNA single-strand breaks and toxicity induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or n-nitrosodimethylamine in hamster and rat liver. *Cancer Lett* **74**, 175-181, doi:10.1016/0304-3835(93)90240-a (1993).
- 161 Asakura, S. *et al.* Effects of dietary restriction on induction of unscheduled DNA synthesis (UDS) and replicative DNA synthesis (RDS) in rat liver. *Mutat Res* **322**, 257-264, doi:10.1016/0165-1218(94)90101-5 (1994).
- 162 Webster, R. P., Gawde, M. D. & Bhattacharya, R. K. Protective effect of rutin, a flavonol glycoside, on the carcinogen-induced DNA damage and repair enzymes in rats. *Cancer Lett* **109**, 185-191, doi:10.1016/s0304-3835(96)04443-6 (1996).
- 163 Mirsalis, J. C. *et al.* Induction of hepatic mutations in lacI transgenic mice. *Mutagenesis* **8**, 265-271, doi:10.1093/mutage/8.3.265 (1993).
- 164 Tinwell, H., Lefevre, P. A. & Ashby, J. Mutation studies with dimethyl nitrosamine in young and old lac I transgenic mice. *Mutat Res* **307**, 501-508, doi:10.1016/0027-5107(94)90261-5 (1994).
- 165 Butterworth, B. E. *et al.* Long-term mutagenicity studies with chloroform and dimethylnitrosamine in female lacI transgenic B6C3F1 mice. *Environ Mol Mutagen* **31**, 248-256, doi:10.1002/(sici)1098-2280(1998)31:3<248::aid-em6>3.0.co;2-g (1998).
- 166 Pool-Zobel, B. L. *et al.* Systemic genotoxic effects of tobacco-related nitrosamines following oral and inhalational administration to Sprague-Dawley rats. *Clin Investig* **70**, 299-306 (1992).
- 167 Diaz Gomez, M. I., Tamayo, D. & Castro, J. A. Administration of N-nitrosodimethylamine, N-nitrosopyrrolidine, or N'-nitroso-nornicotine to nursing rats: their interactions with liver and kidney nucleic acids from sucklings. *J Natl Cancer Inst* **76**, 1133-1136 (1986).
- 168 Hakura, A., Suzuki, S. & Satoh, T. Advantage of the use of human liver S9 in the Ames test. *Mutat Res* **438**, 29-36, doi:10.1016/s1383-5718(98)00159-4 (1999).
- 169 Hakura, A. *et al.* Use of human liver S9 in the Ames test: assay of three procarcinogens using human S9 derived from multiple donors. *Regul Toxicol Pharmacol* **37**, 20-27, doi:10.1016/s0273-2300(02)00024-7 (2003).
- 170 Preston, B. D., Albertson, T. M. & Herr, A. J. DNA replication fidelity and cancer. *Semin Cancer Biol* **20**, 281-293, doi:10.1016/j.semcancer.2010.10.009 (2010).
- 171 Kay, J., Thadhani, E., Samson, L. & Engelward, B. Inflammation-induced DNA damage, mutations and cancer. *DNA Repair (Amst)* **83**, 102673, doi:10.1016/j.dnarep.2019.102673 (2019).
- 172 Souliotis, V. L., Sfikakis, P. P., Anderson, L. M. & Kyrtopoulos, S. A. Intra- and intercellular variations in the repair efficiency of O6-methylguanine, and their contribution to kinetic complexity. *Mutat Res* **568**, 155-170, doi:10.1016/j.mrfmmm.2004.07.009 (2004).
- 173 Hecht, S. S., Trushin, N., Castonguay, A. & Rivenson, A. Comparative tumorigenicity and DNA methylation in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosodimethylamine. *Cancer Res* **46**, 498-502 (1986).

- 174 Dai, W. D. *et al.* DNA methylation in specific cells of rat liver by N-nitrosodimethylamine and N-nitrosomethylbenzylamine. *Carcinogenesis* **12**, 1325-1329, doi:10.1093/carcin/12.7.1325 (1991).
- 175 Chung, F. L., Wang, M. Y. & Hecht, S. S. Effects of dietary indoles and isothiocyanates on N-nitrosodimethylamine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone alpha-hydroxylation and DNA methylation in rat liver. *Carcinogenesis* **6**, 539-543, doi:10.1093/carcin/6.4.539 (1985).
- 176 Hong, J. & Yang, C. S. The nature of microsomal N-nitrosodimethylamine demethylase and its role in carcinogen activation. *Carcinogenesis* **6**, 1805-1809, doi:10.1093/carcin/6.12.1805 (1985).
- 177 Meira, L. B. *et al.* DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. *J Clin Invest* **118**, 2516-2525, doi:10.1172/JCI35073 (2008).
- 178 Klaunig, J. E., Wang, Z., Pu, X. & Zhou, S. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol* **254**, 86-99, doi:10.1016/j.taap.2009.11.028 (2011).
- 179 Ahotupa, M., Bussacchini-Griot, V., Bereziat, J. C., Camus, A. M. & Bartsch, H. Rapid oxidative stress induced by N-nitrosamines. *Biochem Biophys Res Commun* **146**, 1047-1054, doi:10.1016/0006-291x(87)90753-4 (1987).
- 180 Kiziltas, H. *et al.* Antioxidant properties of *Ferulago angulata* and its hepatoprotective effect against N-nitrosodimethylamine-induced oxidative stress in rats. *Pharm Biol* **55**, 888-897, doi:10.1080/13880209.2016.1270974 (2017).
- 181 Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* **357**, 539-545, doi:10.1016/S0140-6736(00)04046-0 (2001).
- 182 Guerra, C. *et al.* Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* **11**, 291-302, doi:10.1016/j.ccr.2007.01.012 (2007).
- 183 Bogen, K. T. Inflammation as a Cancer Co-Initiator: New Mechanistic Model Predicts Low/Negligible Risk at Noninflammatory Carcinogen Doses. *Dose Response* **17**, 1559325819847834, doi:10.1177/1559325819847834 (2019).
- 184 Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860-867 (2002).
- 185 Wang, D. & Dubois, R. N. Eicosanoids and cancer. *Nat Rev Cancer* **10**, 181-193, doi:nrc2809 [pii] 10.1038/nrc2809 (2010).
- 186 Fishbein, A. *et al.* Resolution of eicosanoid/cytokine storm prevents carcinogen and inflammation-initiated hepatocellular cancer progression. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.2007412117 (2020).
- 187 De Cock, J. M. *et al.* Inflammation Triggers Zeb1-Dependent Escape from Tumor Latency. *Cancer Res* **76**, 6778-6784, doi:10.1158/0008-5472.CAN-16-0608 (2016).
- 188 Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K. & Sethi, G. Inflammation and cancer: how hot is the link? *Biochem Pharmacol* **72**, 1605-1621, doi:S0006-2952(06)00389-3 [pii] 10.1016/j.bcp.2006.06.029 (2006).
- 189 Espinoza, J. A. *et al.* The inflammatory inception of gallbladder cancer. *Biochim Biophys Acta* **1865**, 245-254, doi:10.1016/j.bbcan.2016.03.004 (2016).
- 190 Wongsena, W. *et al.* Melatonin suppresses eosinophils and Th17 cells in hamsters treated with a combination of human liver fluke infection and a chemical carcinogen. *Pharmacol Rep* **70**, 98-105, doi:10.1016/j.pharep.2017.07.017 (2018).
- 191 Dangtakot, R. *et al.* CagA(+) *Helicobacter pylori* infection and N-nitrosodimethylamine administration induce cholangiocarcinoma development in hamsters. *Helicobacter*, e12817, doi:10.1111/hel.12817 (2021).
- 192 Yang, H., Wang, H. & Andersson, U. Targeting Inflammation Driven by HMGB1. *Front Immunol* **11**, 484, doi:10.3389/fimmu.2020.00484 (2020).

- 193 Thamavit, W., Kongkanuntn, R., Tiwawech, D. & Moore, M. A. Level of Opisthorchis infestation and carcinogen dose-dependence of cholangiocarcinoma induction in Syrian golden hamsters. *Virchows Arch B Cell Pathol Incl Mol Pathol* **54**, 52-58, doi:10.1007/BF02899196 (1987).
- 194 Boonjaraspinyo, S., Boonmars, T., Aromdee, C., Puapairoj, A. & Wu, Z. Indirect effect of a turmeric diet: enhanced bile duct proliferation in Syrian hamsters with a combination of partial obstruction by Opisthorchis viverrini infection and inflammation by N-nitrosodimethylamine administration. *Parasitol Res* **108**, 7-14, doi:10.1007/s00436-010-2031-7 (2011).
- 195 Boonjaraspinyo, S., Boonmars, T., Aromdee, C. & Kaewsamut, B. Effect of fingerroot on reducing inflammatory cells in hamster infected with Opisthorchis viverrini and N-nitrosodimethylamine administration. *Parasitol Res* **106**, 1485-1489, doi:10.1007/s00436-010-1809-y (2010).
- 196 George, J., Rao, K. R., Stern, R. & Chandrakasan, G. Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* **156**, 129-138 (2001).
- 197 George, J., Tsutsumi, M. & Tsuchishima, M. MMP-13 deletion decreases profibrogenic molecules and attenuates N-nitrosodimethylamine-induced liver injury and fibrosis in mice. *J Cell Mol Med* **21**, 3821-3835, doi:10.1111/jcmm.13304 (2017).
- 198 Kim, E. M., Bae, Y. M., Choi, M. H. & Hong, S. T. Connexin 43 plays an important role in the transformation of cholangiocytes with Clonochis sinensis excretory-secretory protein and N-nitrosodimethylamine. *PLoS Negl Trop Dis* **13**, e0006843, doi:10.1371/journal.pntd.0006843 (2019).
- 199 Desjardins, R., Fournier, M., Denizeau, F. & Krzystyniak, K. Immunosuppression by chronic exposure to N-nitrosodimethylamine (NDMA) in mice. *J Toxicol Environ Health* **37**, 351-361, doi:10.1080/15287399209531676 (1992).
- 200 Jeong, H. G. & Lee, Y. W. Protective effects of diallyl sulfide on N-nitrosodimethylamine-induced immunosuppression in mice. *Cancer Lett* **134**, 73-79, doi:10.1016/s0304-3835(98)00246-8 (1998).
- 201 Ratajczak-Wrona, W. *et al.* PI3K-Akt/PKB signaling pathway in neutrophils and mononuclear cells exposed to N-nitrosodimethylamine. *J Immunotoxicol* **11**, 231-237, doi:10.3109/1547691X.2013.826307 (2014).
- 202 Verna, L., Whysner, J. & Williams, G. M. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacol Ther* **71**, 57-81, doi:10.1016/0163-7258(96)00062-9 (1996).
- 203 Sharma, V. & Singh, M. Attenuation of N-nitrosodimethylamine induced hepatotoxicity by Operculina turpethum in Swiss Albino mice. *Iran J Basic Med Sci* **17**, 73-80 (2014).
- 204 Wang, H. Y., Qin, M., Dong, L., Lv, J. Y. & Wang, X. Genotoxicity of a Low-Dose Nitrosamine Mixture as Drinking Water Disinfection Byproducts in NIH3T3 Cells. *Int J Med Sci* **14**, 961-969, doi:10.7150/ijms.20121 (2017).
- 205 Potten, C. S., Li, Y. Q., O'Connor, P. J. & Winton, D. J. A possible explanation for the differential cancer incidence in the intestine, based on distribution of the cytotoxic effects of carcinogens in the murine large bowel. *Carcinogenesis* **13**, 2305-2312 (1992).
- 206 Li, Y. Q., Fan, C. Y., O'Connor, P. J., Winton, D. J. & Potten, C. S. Target cells for the cytotoxic effects of carcinogens in the murine small bowel. *Carcinogenesis* **13**, 361-368 (1992).
- 207 Kyrtopoulos, S. A. DNA adducts in humans after exposure to methylating agents. *Mutat Res* **405**, 135-143, doi:10.1016/s0027-5107(98)00130-4 (1998).
- 208 Souliotis, V. L., van Delft, J. H., Steenwinkel, M. J., Baan, R. A. & Kyrtopoulos, S. A. DNA adducts, mutant frequencies and mutation spectra in lambda lacZ transgenic mice treated with N-nitrosodimethylamine. *Carcinogenesis* **19**, 731-739 (1998).

- 209 Lin, H. L., Parsels, L. A., Maybaum, J. & Hollenberg, P. F. N-Nitrosodimethylamine-mediated cytotoxicity in a cell line expressing P450 2E1: evidence for apoptotic cell death. *Toxicol Appl Pharmacol* **157**, 117-124, doi:10.1006/taap.1999.8651 (1999).
- 210 Lin, H. & Hollenberg, P. F. N-nitrosodimethylamine-mediated formation of oxidized and methylated dna bases in a cytochrome P450 2E1 expressing cell line. *Chem Res Toxicol* **14**, 562-566, doi:10.1021/tx0001979 (2001).
- 211 Suksawat, M. *et al.* Upregulation of endothelial nitric oxide synthase (eNOS) and its upstream regulators in *Opisthorchis viverrini* associated cholangiocarcinoma and its clinical significance. *Parasitol Int* **66**, 486-493, doi:10.1016/j.parint.2016.04.008 (2017).
- 212 Iwaniuk, A., Jablonska, E., Jablonski, J., Ratajczak-Wrona, W. & Garley, M. Expression of selected proteins of the extrinsic and intrinsic pathways of apoptosis in human leukocytes exposed to N-nitrosodimethylamine. *Hum Exp Toxicol* **34**, 591-600, doi:10.1177/0960327114551391 (2015).
- 213 Gombar, C. T. *et al.* Interspecies scaling of the pharmacokinetics of N-nitrosodimethylamine. *Cancer Res* **50**, 4366-4370 (1990).
- 214 Gombar, C. T. *et al.* Pharmacokinetics of N-nitrosodimethylamine in swine. *Carcinogenesis* **9**, 1351-1354, doi:10.1093/carcin/9.8.1351 (1988).
- 215 Gombar, C. T., Pylypiw, H. M., Jr. & Harrington, G. W. Pharmacokinetics of N-nitrosodimethylamine in beagles. *Cancer Res* **47**, 343-347 (1987).
- 216 Streeter, A. J. *et al.* Metabolic denitrosation of N-nitrosodimethylamine in vivo in the rat. *Cancer Res* **50**, 1144-1150 (1990).
- 217 Clapp, N. K., Craig, A. W. & Toya, R. E., Sr. Diethylnitrosamine oncogenesis in RF mice as influenced by variations in cumulative dose. *Int J Cancer* **5**, 119-123, doi:10.1002/ijc.2910050116 (1970).
- 218 Rajewsky, M. F., Dauber, W. & Frankenberg, H. Liver carcinogenesis by diethylnitrosamine in the rat. *Science* **152**, 83-85, doi:10.1126/science.152.3718.83 (1966).
- 219 Arcos, J. C., Argus, M. F. & Mathison, J. B. Hepatic carcinogenesis threshold and biphasic mitochondrial swelling response in the guinea-pig during diethylnitrosamine administration. *Experientia* **25**, 296-298, doi:10.1007/BF02034405 (1969).
- 220 Montesano, R. & Saffiotti, U. Carcinogenic response of the respiratory tract of Syrian golden hamsters to different doses of diethylnitrosamine. *Cancer Res* **28**, 2197-2210 (1968).
- 221 Argus, M. F. & Hoch-Ligeti, C. Induction of malignant tumors in the guinea pig by oral administration of diethylnitrosamine. *J Natl Cancer Inst* **30**, 533-551 (1963).
- 222 Lijinsky, W., Reuber, M. D. & Riggs, C. W. Dose response studies of carcinogenesis in rats by nitrosodiethylamine. *Cancer Res* **41**, 4997-5003 (1981).
- 223 Hendricks, J. D., Cheng, R., Shelton, D. W., Pereira, C. B. & Bailey, G. S. Dose-dependent carcinogenicity and frequent Ki-ras proto-oncogene activation by dietary N-nitrosodiethylamine in rainbow trout. *Fundam Appl Toxicol* **23**, 53-62, doi:10.1006/faat.1994.1078 (1994).
- 224 Thorgeirsson, U. P., Dalgard, D. W., Reeves, J. & Adamson, R. H. Tumor incidence in a chemical carcinogenesis study of nonhuman primates. *Regul Toxicol Pharmacol* **19**, 130-151, doi:10.1006/rtph.1994.1013 (1994).
- 225 Berger, M. R., Schmahl, D. & Zurban, H. Combination experiments with very low doses of three genotoxic N-nitrosamines with similar organotropic carcinogenicity in rats. *Carcinogenesis* **8**, 1635-1643, doi:10.1093/carcin/8.11.1635 (1987).
- 226 Hidajat, M. *et al.* Lifetime exposure to rubber dusts, fumes and N-nitrosamines and cancer mortality in a cohort of British rubber workers with 49 years follow-up. *Occup Environ Med* **76**, 250-258, doi:10.1136/oemed-2018-105181 (2019).

- 227 De Stefani, E., Boffetta, P., Mendilaharsu, M., Carzoglio, J. & Deneo-Pellegrini, H. Dietary nitrosamines, heterocyclic amines, and risk of gastric cancer: a case-control study in Uruguay. *Nutr Cancer* **30**, 158-162, doi:10.1080/01635589809514656 (1998).
- 228 Pobel, D., Riboli, E., Cornee, J., Hemon, B. & Guyader, M. Nitrosamine, nitrate and nitrite in relation to gastric cancer: a case-control study in Marseille, France. *Eur J Epidemiol* **11**, 67-73 (1995).
- 229 La Vecchia, C., D'Avanzo, B., Airoldi, L., Braga, C. & Decarli, A. Nitrosamine intake and gastric cancer risk. *Eur J Cancer Prev* **4**, 469-474 (1995).
- 230 Zhu, Y. *et al.* Dietary N-nitroso compounds and risk of colorectal cancer: a case-control study in Newfoundland and Labrador and Ontario, Canada. *Br J Nutr* **111**, 1109-1117, doi:10.1017/S0007114513003462 (2014).
- 231 Larsson, S. C., Bergkvist, L. & Wolk, A. Processed meat consumption, dietary nitrosamines and stomach cancer risk in a cohort of Swedish women. *Int J Cancer* **119**, 915-919, doi:10.1002/ijc.21925 (2006).
- 232 Keszei, A. P., Goldbohm, R. A., Schouten, L. J., Jakszyn, P. & van den Brandt, P. A. Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study. *Am J Clin Nutr* **97**, 135-146, doi:10.3945/ajcn.112.043885 (2013).
- 233 Loh, Y. H. *et al.* N-Nitroso compounds and cancer incidence: the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk Study. *Am J Clin Nutr* **93**, 1053-1061, doi:10.3945/ajcn.111.012377 (2011).
- 234 Zheng, J. *et al.* Dietary N-nitroso compounds and risk of pancreatic cancer: results from a large case-control study. *Carcinogenesis* **40**, 254-262, doi:10.1093/carcin/bgy169 (2019).
- 235 Goodman, M. T., Hankin, J. H., Wilkens, L. R. & Kolonel, L. N. High-fat foods and the risk of lung cancer. *Epidemiology* **3**, 288-299 (1992).
- 236 Knekt, P., Jarvinen, R., Dich, J. & Hakulinen, T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. *Int J Cancer* **80**, 852-856 (1999).
- 237 Kirsch-Volders, M., Aardema, M. & Elhajouji, A. Concepts of threshold in mutagenesis and carcinogenesis. *Mutat Res* **464**, 3-11, doi:10.1016/s1383-5718(99)00161-8 (2000).
- 238 Anderson, L. M., Hagiwara, A., Kovatch, R. M., Rehm, S. & Rice, J. M. Transplacental initiation of liver, lung, neurogenic, and connective tissue tumors by N-nitroso compounds in mice. *Fundam Appl Toxicol* **12**, 604-620, doi:10.1016/0272-0590(89)90033-x (1989).
- 239 Tielemans, E. *et al.* Conceptual model for assessment of inhalation exposure: defining modifying factors. *Ann Occup Hyg* **52**, 577-586, doi:10.1093/annhyg/men059 (2008).
- 240 Hidajat, M. *et al.* Job-exposure matrix for historical exposures to rubber dust, rubber fumes and n-Nitrosamines in the British rubber industry. *Occup Environ Med* **76**, 259-267, doi:10.1136/oemed-2018-105182 (2019).
- 241 Cherrie, J. W., McElvenny, D. & Blyth, K. G. Estimating past inhalation exposure to asbestos: A tool for risk attribution and disease screening. *Int J Hyg Environ Health* **221**, 27-32, doi:10.1016/j.ijheh.2017.09.013 (2018).
- 242 Klein, R. G. & Schmezer, P. Quantitative measurement of the exhalation rate of volatile N-nitrosamines in inhalation experiments with anaesthetized Sprague-Dawley rats. *IARC Sci Publ*, 513-517 (1984).
- 243 Lutz, W. K. Dose-response relationships in chemical carcinogenesis reflect differences in individual susceptibility. Consequences for cancer risk assessment, extrapolation, and prevention. *Hum Exp Toxicol* **18**, 707-712, doi:10.1191/096032799678839653 (1999).

- 244 Hill, A. B. The environment and disease: association or causation? 1965. *J R Soc Med* **108**, 32-37, doi:10.1177/0141076814562718 (2015).
- 245 Pottegard, A. *et al.* Use of N-nitrosodimethylamine (NDMA) contaminated valsartan products and risk of cancer: Danish nationwide cohort study. *BMJ* **362**, k3851, doi:10.1136/bmj.k3851 (2018).
- 246 Gomm, W. *et al.* N-Nitrosodimethylamine-Contaminated Valsartan and the Risk of Cancer-A Longitudinal Cohort Study Based on German Health Insurance Data. *Dtsch Arztebl Int* **118**, doi:10.3238/arztebl.m2021.0129 (2021).
- 247 Mohr, U., Haas, H. & Hilfrich, J. The carcinogenic effects of dimethylnitrosamine and nitrosomethylurea in European hamsters (*Cricetus cricetus* L.). *Br J Cancer* **29**, 359-364, doi:10.1038/bjc.1974.83 (1974).
- 248 Anderson, L. M., Ward, J. M., Fox, S. D., Isaaq, H. J. & Riggs, C. W. Effects of a single dose of polychlorinated biphenyls to infant mice on N-nitrosodimethylamine-initiated lung and liver tumors. *Int J Cancer* **38**, 109-116 (1986).
- 249 Toth, B., Magee, P. N. & Shubik, P. Carcinogenesis Study with Dimethylnitrosamine Administered Orally to Adult and Subcutaneously to Newborn Balb-C Mice. *Cancer Res* **24**, 1712-1721 (1964).
- 250 Hoch-Ligeti, C., Argus, M. F. & Arcos, J. C. Combined carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene in the rat. *J Natl Cancer Inst* **40**, 535-549 (1968).
- 251 Keefer, L. K., Lijinsky, W. & Garcia, H. Deuterium isotope effect on the carcinogenicity of dimethylnitrosamine in rat liver. *J Natl Cancer Inst* **51**, 299-302, doi:10.1093/jnci/51.1.299 (1973).
- 252 Takayama, S. & Oota, K. Malignant Tumors Induced in Mice Fed with N-Nitrosodimethylamine. *Gan* **54**, 465-472 (1963).
- 253 Takayama, S. & Oota, K. Induction of Malignant Tumors in Various Strains of Mice by Oral Administration of N-Nitrosodimethylamine and N-Nitrosodiethylamine. *Gan* **56**, 189-199 (1965).
- 254 Den Engelse, L. The formation of methylated bases in DNA by dimethylnitrosamine and its relation to differences in the formation of tumours in the livers of GR and C3HF mice. *Chem Biol Interact* **8**, 329-338, doi:10.1016/0009-2797(74)90011-8 (1974).
- 255 den Engelse, L., Bentvelzen, P. A. & Emmelot, P. Studies on lung tumours. I. Methylation of deoxyribonucleic acid and tumour formation following administration of dimethylnitrosamine to mice. *Chem Biol Interact* **1**, 395-406, doi:10.1016/0009-2797(70)90006-2 (1970).
- 256 Clapp, N. K. & Craig, A. W. Carcinogenic effects of diethylnitrosamine in RF mice. *J Natl Cancer Inst* **39**, 903-916 (1967).
- 257 Vesselinovitch, S. D. The sex-dependent difference in the development of liver tumors in mice administered dimethylnitrosamine. *Cancer Res* **29**, 1024-1027 (1969).
- 258 Ingram, A. J. The lethal and hepatocarcinogenic effects of dimethylnitrosamine injection in the newt *Triturus helveticus*. *Br J Cancer* **26**, 206-215, doi:10.1038/bjc.1972.28 (1972).
- 259 Ashley, L. M. & Halver, J. E. Dimethylnitrosamine-induced hepatic cell carcinoma in rainbow trout. *J Natl Cancer Inst* **41**, 531-552 (1968).
- 260 Sato, S., Matsushima, T., Tanaka, N., Sugimura, T. & Takashima, F. Hepatic tumors in the guppy (*Lebistes reticulatus*) induced by aflatoxin B 1 , dimethylintrosamine, and 2-acetylaminofluorene. *J Natl Cancer Inst* **50**, 767-778, doi:10.1093/jnci/50.3.767 (1973).
- 261 Delgado, M. E., Haza, A. I., Arranz, N., Garcia, A. & Morales, P. Dietary polyphenols protect against N-nitrosamines and benzo(a)pyrene-induced DNA damage (strand breaks and oxidized purines/pyrimidines) in HepG2 human hepatoma cells. *Eur J Nutr* **47**, 479-490, doi:10.1007/s00394-008-0751-6 (2008).
- 262 Yoo, J. S., Guengerich, F. P. & Yang, C. S. Metabolism of N-nitrosodialkylamines by human liver microsomes. *Cancer Res* **48**, 1499-1504 (1988).

- 263 Yamazaki, H., Inui, Y., Yun, C. H., Guengerich, F. P. & Shimada, T. Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of N-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. *Carcinogenesis* **13**, 1789-1794, doi:10.1093/carcin/13.10.1789 (1992).
- 264 Patten, C. J. *et al.* Catalytic properties of the human cytochrome P450 2E1 produced by cDNA expression in mammalian cells. *Arch Biochem Biophys* **299**, 163-171, doi:10.1016/0003-9861(92)90258-x (1992).
- 265 Erkekoglu, P. & Baydar, T. Evaluation of the protective effect of ascorbic acid on nitrite- and nitrosamine-induced cytotoxicity and genotoxicity in human hepatoma line. *Toxicol Mech Methods* **20**, 45-52, doi:10.3109/15376510903583711 (2010).
- 266 Chao, M. R., Chang, Y. Z., Wong, R. H. & Hu, C. W. Time course evaluation of N-nitrosodialkylamines-induced DNA alkylation and oxidation in liver of mosquito fish. *Mutat Res* **660**, 33-39, doi:10.1016/j.mrfmmm.2008.10.004 (2009).
- 267 Dechakhamphu, S., Pinlaor, S., Sitthithaworn, P., Bartsch, H. & Yongvanit, P. Accumulation of miscoding etheno-DNA adducts and highly expressed DNA repair during liver fluke-induced cholangiocarcinogenesis in hamsters. *Mutat Res* **691**, 9-16, doi:10.1016/j.mrfmmm.2010.06.003 (2010).
- 268 Martino, P. E., Diaz Gomez, M. I., Tamayo, D., Lopez, A. J. & Castro, J. A. Studies on the mechanism of the acute and carcinogenic effects of N-nitrosodimethylamine on mink liver. *J Toxicol Environ Health* **23**, 183-192, doi:10.1080/15287398809531105 (1988).
- 269 Hakkola, J., Tanaka, E. & Pelkonen, O. Developmental expression of cytochrome P450 enzymes in human liver. *Pharmacol Toxicol* **82**, 209-217, doi:10.1111/j.1600-0773.1998.tb01427.x (1998).
- 270 Chala, B. *et al.* Development of Urinary Bladder Pre-Neoplasia by Schistosoma haematobium Eggs and Chemical Carcinogen in Mice. *Korean J Parasitol* **55**, 21-29, doi:10.3347/kjp.2017.55.1.21 (2017).
- 271 Ding, X. *et al.* Expression of HMGA2 in bladder cancer and its association with epithelial-to-mesenchymal transition. *Cell Prolif* **47**, 146-151, doi:10.1111/cpr.12096 (2014).
- 272 Sheweita, S. A., El-Shahat, F. G., Bazeed, M. A., Abu El-Maati, M. R. & O'Connor, P. J. Effects of Schistosoma haematobium infection on drug-metabolizing enzymes in human bladder cancer tissues. *Cancer Lett* **205**, 15-21, doi:10.1016/j.canlet.2003.09.023 (2004).
- 273 Al-Saireh, Y. M. *et al.* Profiling of CYP4Z1 and CYP1B1 expression in bladder cancers. *Sci Rep* **11**, 5581, doi:10.1038/s41598-021-85188-4 (2021).
- 274 Iwaniuk, A. *et al.* N-nitrosodimethylamine (NDMA) induced apoptosis dependent on Fas/FasL complex in human leukocytes. *Hum Exp Toxicol* **38**, 578-587, doi:10.1177/0960327119828198 (2019).
- 275 Ratajczak-Wrona, W., Jablonska, E., Jablonski, J. & Marcinczyk, M. Induction of expression of iNOS by N-nitrosodimethylamine (NDMA) in human leukocytes. *Immunopharmacol Immunotoxicol* **31**, 661-668, doi:10.3109/08923970903014984 (2009).
- 276 Dobo, K. L., Eastmond, D. A. & Grososky, A. J. Sequence specific mutations induced by N-nitrosodimethylamine at two marker loci in metabolically competent human lymphoblastoid cells. *Carcinogenesis* **19**, 755-764, doi:10.1093/carcin/19.5.755 (1998).
- 277 Furukawa, M. *et al.* Cytochrome p450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. *Cancer Sci* **95**, 520-529, doi:10.1111/j.1349-7006.2004.tb03243.x (2004).
- 278 Nagai, F., Hiyoshi, Y., Sugimachi, K. & Tamura, H. O. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. *Biol Pharm Bull* **25**, 383-385, doi:10.1248/bpb.25.383 (2002).

- 279 Preston-Martin, S. *et al.* Maternal consumption of cured meats and vitamins in relation to pediatric brain tumors. *Cancer Epidemiol Biomarkers Prev* **5**, 599-605 (1996).
- 280 Huncharek, M. & Kupelnick, B. A meta-analysis of maternal cured meat consumption during pregnancy and the risk of childhood brain tumors. *Neuroepidemiology* **23**, 78-84, doi:10.1159/000073979 (2004).
- 281 Dietrich, M. *et al.* A review: dietary and endogenously formed N-nitroso compounds and risk of childhood brain tumors. *Cancer Causes Control* **16**, 619-635, doi:10.1007/s10552-005-0168-y (2005).
- 282 Murray, G. I., Taylor, M. C., Burke, M. D. & Melvin, W. T. Enhanced expression of cytochrome P450 in stomach cancer. *Br J Cancer* **77**, 1040-1044, doi:10.1038/bjc.1998.173 (1998).
- 283 Krul, C. A., Zeilmaier, M. J., Schothorst, R. C. & Havenaar, R. Intragastric formation and modulation of N-nitrosodimethylamine in a dynamic in vitro gastrointestinal model under human physiological conditions. *Food Chem Toxicol* **42**, 51-63, doi:10.1016/j.fct.2003.08.005 (2004).
- 284 Hebels, D. G., Jennen, D. G., Kleinjans, J. C. & de Kok, T. M. Molecular signatures of N-nitroso compounds in Caco-2 cells: implications for colon carcinogenesis. *Toxicol Sci* **108**, 290-300, doi:10.1093/toxsci/kfp035 (2009).
- 285 Autrup, H. *et al.* Explant culture of rat colon: a model system for studying metabolism of chemical carcinogens. *In Vitro* **14**, 868-877 (1978).
- 286 Thelen, K. & Dressman, J. B. Cytochrome P450-mediated metabolism in the human gut wall. *J Pharm Pharmacol* **61**, 541-558, doi:10.1211/jpp/61.05.0002 (2009).
- 287 Parsa, I., Marsh, W. H. & Sutton, A. L. An in vitro model of human pancreas carcinogenesis: effects of nitroso compounds. *Cancer* **47**, 1543-1551, doi:10.1002/1097-0142(19810315)47:6+<1543::aid-cnrcr2820471417>3.0.co;2-3 (1981).
- 288 Parsa, I., Marsh, W. H., Sutton, A. L. & Butt, K. M. Effects of dimethylnitrosamine on organ-cultured adult human pancreas. *Am J Pathol* **102**, 403-411 (1981).
- 289 Parsa, I., Friedman, S. & Cleary, C. M. Visualization of O6-methylguanine in target cell nuclei of dimethylnitrosamine-treated human pancreas by a murine monoclonal antibody. *Carcinogenesis* **8**, 839-846, doi:10.1093/carcin/8.6.839 (1987).
- 290 Guo, Y., Zhu, L. R., Lu, G., Wang, H. & Hong, J. Y. Selective expression of CYP2A13 in human pancreatic alpha-islet cells. *Drug Metab Dispos* **40**, 1878-1882, doi:10.1124/dmd.112.046359 (2012).
- 291 Wacke, R. *et al.* Up-regulation of cytochrome P450 1A2, 2C9, and 2E1 in chronic pancreatitis. *Pancreas* **16**, 521-528, doi:10.1097/00006676-199805000-00011 (1998).
- 292 Smith, T. J. *et al.* Characterization of xenobiotic-metabolizing enzymes and nitrosamine metabolism in the human esophagus. *Carcinogenesis* **19**, 667-672, doi:10.1093/carcin/19.4.667 (1998).
- 293 Autrup, H. & Stoner, G. D. Metabolism of N-nitrosamines by cultured human and rat esophagus. *Cancer Res* **42**, 1307-1311 (1982).
- 294 Maksymchuk, O. V. & Kashuba, V. I. Altered expression of cytochrome P450 enzymes involved in metabolism of androgens and vitamin D in the prostate as a risk factor for prostate cancer. *Pharmacol Rep* **72**, 1161-1172, doi:10.1007/s43440-020-00133-y (2020).
- 295 Zak, F. G., Holzner, J. H., Singer, E. J. & Popper, H. Renal and pulmonary tumors in rats fed dimethylnitrosamine. *Cancer Res* **20**, 96-99 (1960).
- 296 Noronha, R. F. The inhibition of dimethylnitrosamine-induced renal tumorigenesis in NZO/Bl mice by orchiectomy. *Invest Urol* **13**, 136-141 (1975).
- 297 Clapp, N. K. Carcinogenicity of nitrosamines and methanesulphonate esters given intraperitoneally, in RF mice. *Int J Cancer* **12**, 728-733, doi:10.1002/ijc.2910120320 (1973).

- 298 Cardesa, Y. I., Patil, K., Althoff, J. & Pour, P. Comparative studies of neoplastic response to a single dose of nitroso compounds. 5. The effect of dimethylnitrosamine in Swiss, ASW/SN and A-strain mice. *Z Krebsforsch Klin Onkol Cancer Res Clin Oncol* **86**, 165-170, doi:10.1007/BF00284004 (1976).
- 299 Cardesa, A., Pour, P., Althoff, J. & Mohr, U. Comparative studies of neoplastic response to a single dose of nitroso compounds. 4. The effect of dimethyl- and diethyl-nitrosamine in Swiss mice. *Z Krebsforsch Klin Onkol Cancer Res Clin Oncol* **81**, 229-233, doi:10.1007/BF00305023 (1974).
- 300 Argus, M. F. & Hoch-Ligeti, C. Comparative study of the carcinogenic activity of nitrosamines. *J Natl Cancer Inst* **27**, 695-709 (1961).
- 301 den Engelse, L., Gebbink, M. & Emmelot, P. Studies on lung tumours. III. Oxidative metabolism of dimethylnitrosamine by rodent and human lung tissue. *Chem Biol Interact* **11**, 535-544, doi:10.1016/0009-2797(75)90029-0 (1975).
- 302 Anttila, S., Raunio, H. & Hakkola, J. Cytochrome P450-mediated pulmonary metabolism of carcinogens: regulation and cross-talk in lung carcinogenesis. *Am J Respir Cell Mol Biol* **44**, 583-590, doi:10.1165/rcmb.2010-0189RT (2011).
- 303 Magee, P. N. & Barnes, J. M. The experimental production of tumours in the rat by dimethylnitrosamine (N-nitroso dimethylamine). *Acta Unio Int Contra Cancrum* **15**, 187-190 (1959).
- 304 Riopelle, J. L. & Jasmin, G. Nature, classification, and nomenclature of kidney tumors induced in the rat by dimethylnitrosamine. *J Natl Cancer Inst* **42**, 643-662 (1969).
- 305 Shinohara, Y., Arai, M., Hirao, K., Sugihara, S. & Nakanishi, K. Combination effect of citrinin and other chemicals on rat kidney tumorigenesis. *Gan* **67**, 147-155 (1976).
- 306 Swann, P. F., Kaufman, D. G., Magee, P. N. & Mace, R. Induction of kidney tumours by a single dose of dimethylnitrosamine: dose response and influence of diet and benzo(a)pyrene pretreatment. *Br J Cancer* **41**, 285-294, doi:10.1038/bjc.1980.41 (1980).
- 307 McGiven, A. R. & Ireton, H. J. Renal epithelial dysplasia and neoplasia in rats given dimethylnitrosamine. *J Pathol* **108**, 187-190, doi:10.1002/path.1711080303 (1972).
- 308 Ireton, H. J., McGiven, A. R. & Davies, D. J. Renal mesenchymal tumours induced in rats by dimethylnitrosamine: light- and electron-microscope studies. *J Pathol* **108**, 181-185, doi:10.1002/path.1711080302 (1972).
- 309 McLean, A. E. & Magee, P. N. Increased renal carcinogenesis by dimethyl nitrosamine in protein deficient rats. *Br J Exp Pathol* **51**, 587-590 (1970).
- 310 Montesano, R., Mohr, U., Magee, P. N., Hilfrich, J. & Haas, H. Additive effect in the induction of kidney tumours in rats treated with dimethylnitrosamine and ethylmethanesulphonate. *Br J Cancer* **29**, 50-58, doi:10.1038/bjc.1974.6 (1974).
- 311 Murphy, G. P., Mirand, E. A., Johnston, G. S., Schmidt, J. D. & Scott, W. W. Renal tumors induced by a single dose of dimethylnitrosamine: morphologic, functional, enzymatic, and hormonal characterizations. *Invest Urol* **4**, 39-56 (1966).
- 312 Hard, G. C. & Butler, W. H. Morphogenesis of epithelial neoplasms induced in the rat kidney by dimethylnitrosamine. *Cancer Res* **31**, 1496-1505 (1971).
- 313 Hard, G. C. & Butler, W. H. Ultrastructural aspects of renal adenocarcinoma induced in the rat by dimethylnitrosamine. *Cancer Res* **31**, 366-372 (1971).
- 314 Ito, N. Experimental studies on tumors of the urinary system of rats induced by chemical carcinogens. *Acta Pathol Jpn* **23**, 87-109, doi:10.1111/j.1440-1827.1973.tb00777.x (1973).
- 315 Druckrey, H., Schildbach, A., Schmaehl, D., Preussmann, R. & Ivankovic, S. [Quantitative Analysis of the Carcinogenic Effect of Diethylnitrosamine]. *Arzneimittelforschung* **13**, 841-851 (1963).

- 316 Driver, H. E., White, I. N., Steven, F. S. & Butler, W. H. A possible mechanism for the dose-response relationship observed for renal mesenchymal tumours induced in the rat by a single dose of N-nitrosodimethylamine. *IARC Sci Publ*, 253-255 (1987).
- 317 Aiub, C. A. *et al.* N-Nitrosodiethylamine genotoxicity in primary rat hepatocytes: effects of cytochrome P450 induction by phenobarbital. *Toxicol Lett* **206**, 139-143, doi:10.1016/j.toxlet.2011.07.002 (2011).
- 318 de Rainho, C. R., Kaezer, A., Aiub, C. A. & Felzenszwalb, I. Ability of *Allium cepa* L. root tips and *Tradescantia pallida* var. *purpurea* in N-nitrosodiethylamine genotoxicity and mutagenicity evaluation. *An Acad Bras Cienc* **82**, 925-932, doi:10.1590/s0001-37652010000400015 (2010).
- 319 Westphal, G. A., Bunger, J., Schulz, T. G., Muller, M. M. & Hallier, E. Mutagenicity of N-nitrosodiethylamine in the Ames test with *S. typhimurium* TA1535 is due to volatile metabolites and is not dependent on cytochrome P450E1 induction. *Arch Toxicol* **74**, 638-641, doi:10.1007/s002040000175 (2000).
- 320 Stuff, J. E., Goh, E. T., Barrera, S. L., Bondy, M. L. & Forman, M. R. Construction of an N-nitroso database for assessing dietary intake. *J Food Compos Anal* **22**, S42-S47, doi:10.1016/j.jfca.2009.01.008 (2009).
- 321 Tricker, A. R. & Preussmann, R. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutat Res* **259**, 277-289 (1991).
- 322 Gopalakrishnan, R., Sundaram, J., Sattu, K., Pandi, A. & Thiruvengadam, D. Dietary supplementation of silymarin is associated with decreased cell proliferation, increased apoptosis, and activation of detoxification system in hepatocellular carcinoma. *Mol Cell Biochem* **377**, 163-176, doi:10.1007/s11010-013-1582-1 (2013).
- 323 Abdel-Hamid, N. M., Nazmy, M. H., Abdel-Ghany, M. I. & Nazmy, W. H. Cytokines as important playmakers of experimental hepatocarcinogenesis confounded by diabetes. *Ann Hepatol* **11**, 118-127 (2012).
- 324 Saha, B. K., Sarkar, A., Basak, R. & Chatterjee, M. 1alpha, 25-Dihydroxyvitamin D3 suppresses the effect of streptozotocin-induced diabetes during chemical rat liver carcinogenesis. *Cell Biol Int* **25**, 227-237, doi:10.1006/cbir.2000.0625 (2001).
- 325 Akshatha, G. M., Raval, S. K., Arpitha, G. M., Raval, S. H. & Ghodasara, D. J. Immunohistochemical, histopathological study and chemoprotective effect of *Solanum nigrum* in N-nitrosodiethylamine-induced hepatocellular carcinoma in Wistar rats. *Vet World* **11**, 402-409, doi:10.14202/vetworld.2018.402-409 (2018).
- 326 Diwan, B. A., Henneman, J. R. & Nims, R. W. Enhancement of N-nitrosodiethylamine-initiated hepatocarcinogenesis by phentoin in male F344/NCr rats at a dose causing maximal induction of CYP2B. *Int J Toxicol* **20**, 81-87, doi:10.1080/10915810151115191 (2001).
- 327 Beebe, L. E., Fornwald, L. W., Diwan, B. A., Anver, M. R. & Anderson, L. M. Promotion of N-nitrosodiethylamine-initiated hepatocellular tumors and hepatoblastomas by 2,3,7,8-tetrachlorodibenzo-p-dioxin or Aroclor 1254 in C57BL/6, DBA/2, and B6D2F1 mice. *Cancer Res* **55**, 4875-4880 (1995).
- 328 Diwan, B. A. *et al.* Effects of the oxazolidinedione anticonvulsants trimethadione and dimethadione and the barbiturate homolog 5,5-dimethylbarbituric acid on N-nitrosodiethylamine-initiated renal and hepatic carcinogenesis in the F344/NCr rat. *Arch Toxicol* **66**, 413-422, doi:10.1007/BF02035132 (1992).
- 329 Diwan, B. A., Henneman, J. R., Nims, R. W. & Rice, J. M. Tumor promotion by an anticonvulsant agent, phenytoin, in mouse liver: correlation with CYP2B induction. *Carcinogenesis* **14**, 2227-2231, doi:10.1093/carcin/14.11.2227 (1993).
- 330 Diwan, B. A. *et al.* Lack of promoting effect of clonazepam on the development of N-nitrosodiethylamine-initiated hepatocellular tumors in mice is correlated with its inability to

- inhibit cell-to-cell communication in mouse hepatocytes. *Carcinogenesis* **10**, 1719-1724, doi:10.1093/carcin/10.9.1719 (1989).
- 331 Diwan, B. A., Lubet, R. A., Ward, J. M., Hrabie, J. A. & Rice, J. M. Tumor-promoting and hepatocarcinogenic effects of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in DBA/2Ncr and C57BL/6Ncr mice and an apparent promoting effect on nasal cavity tumors but not on hepatocellular tumors in F344/Ncr rats initiated with N-nitrosodiethylamine. *Carcinogenesis* **13**, 1893-1901, doi:10.1093/carcin/13.10.1893 (1992).
- 332 Diwan, B. A., Ward, J. M., Kurata, Y. & Rice, J. M. Dissimilar frequency of hepatoblastomas and hepatic cystadenomas and adenocarcinomas arising in hepatocellular neoplasms of D2B6F1 mice initiated with N-nitrosodiethylamine and subsequently given Aroclor-1254, dichlorodiphenyltrichloroethane, or phenobarbital. *Toxicol Pathol* **22**, 430-439, doi:10.1177/019262339402200409 (1994).
- 333 Diwan, B. A., Henneman, J. R. & Rice, J. M. Further evidence for promoter-dependent development of hepatoblastoma in the mouse. *Cancer Lett* **89**, 29-35, doi:10.1016/0304-3835(95)90154-x (1995).
- 334 Diwan, B. A., Henneman, J. R., Rice, J. M. & Nims, R. W. Enhancement of thyroid and hepatocarcinogenesis by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene in rats at doses that cause maximal induction of CYP2B. *Carcinogenesis* **17**, 37-43, doi:10.1093/carcin/17.1.37 (1996).
- 335 Dragani, T. A., Manenti, G. & Della Porta, G. Genetic susceptibility to murine hepatocarcinogenesis is associated with high growth rate of NDEA-initiated hepatocytes. *J Cancer Res Clin Oncol* **113**, 223-229, doi:10.1007/BF00396377 (1987).
- 336 Dragani, T. A., Manenti, G. & Della Porta, G. Enhancing effects of carbon tetrachloride in mouse hepatocarcinogenesis. *Cancer Lett* **31**, 171-179, doi:10.1016/0304-3835(86)90008-x (1986).
- 337 Ghia, M., Mattioli, F. & Mereto, E. A possible medium-term assay for detecting the effects of liver and colon carcinogens in rats. *Cancer Lett* **105**, 71-75, doi:10.1016/0304-3835(96)04267-x (1996).
- 338 Hussain, T., Siddiqui, H. H., Fareed, S., Vijayakumar, M. & Rao, C. V. Chemopreventive evaluation of Tephrosia purpurea against N-nitrosodiethylamine-induced hepatocarcinogenesis in Wistar rats. *J Pharm Pharmacol* **64**, 1195-1205, doi:10.1111/j.2042-7158.2012.01503.x (2012).
- 339 Hussain, T., Siddiqui, H. H., Fareed, S., Vijayakumar, M. & Rao, C. V. Evaluation of chemopreventive effect of Fumaria indica against N-nitrosodiethylamine and CCl4-induced hepatocellular carcinoma in Wistar rats. *Asian Pac J Trop Med* **5**, 623-629, doi:10.1016/S1995-7645(12)60128-X (2012).
- 340 Keshari, A. K. *et al.* 5H-benzo[h]thiazolo[2,3-b]quinazolines ameliorate NDEA-induced hepatocellular carcinogenesis in rats through IL-6 downregulation along with oxidative and metabolic stress reduction. *Drug Des Devel Ther* **11**, 2981-2995, doi:10.2147/DDDT.S143075 (2017).
- 341 Sivaramakrishnan, V., Shilpa, P. N., Praveen Kumar, V. R. & Niranjali Devaraj, S. Attenuation of N-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol-Morin. *Chem Biol Interact* **171**, 79-88, doi:10.1016/j.cbi.2007.09.003 (2008).
- 342 Alghzzawy, Z. M., Elmaghraby, T. K., El-Hamid Hagag, S. A. & Awwad, M. H. Combretastatin A-4 disodium phosphate and low dose gamma irradiation suppress hepatocellular carcinoma by downregulating ROCK1 and VEGF gene expression. *Mol Biol Rep* **47**, 1883-1893, doi:10.1007/s11033-020-05282-0 (2020).
- 343 Anis, K. V., Rajeshkumar, N. V. & Kuttan, R. Inhibition of chemical carcinogenesis by berberine in rats and mice. *J Pharm Pharmacol* **53**, 763-768, doi:10.1211/0022357011775901 (2001).

- 344 Jeena, K. J., Joy, K. L. & Kuttan, R. Effect of *Embllica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer Lett* **136**, 11-16, doi:10.1016/s0304-3835(98)00294-8 (1999).
- 345 Arul, D. & Subramanian, P. Inhibitory effect of naringenin (citrus flavonone) on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *Biochem Biophys Res Commun* **434**, 203-209, doi:10.1016/j.bbrc.2013.03.039 (2013).
- 346 Sivaramakrishnan, V. & Devaraj, S. N. Morin fosters apoptosis in experimental hepatocellular carcinogenesis model. *Chem Biol Interact* **183**, 284-292, doi:10.1016/j.cbi.2009.11.011 (2010).
- 347 Singh, B. N., Singh, B. R., Sarma, B. K. & Singh, H. B. Potential chemoprevention of N-nitrosodiethylamine-induced hepatocarcinogenesis by polyphenolics from *Acacia nilotica* bark. *Chem Biol Interact* **181**, 20-28, doi:10.1016/j.cbi.2009.05.007 (2009).
- 348 Ramakrishnan, G., Raghavendran, H. R., Vinodhkumar, R. & Devaki, T. Suppression of N-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats. *Chem Biol Interact* **161**, 104-114, doi:10.1016/j.cbi.2006.03.007 (2006).
- 349 Ramakrishnan, G., Augustine, T. A., Jagan, S., Vinodhkumar, R. & Devaki, T. Effect of silymarin on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *Exp Oncol* **29**, 39-44 (2007).
- 350 Khanduja, K. L., Gandhi, R. K., Pathania, V. & Syal, N. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. *Food Chem Toxicol* **37**, 313-318, doi:10.1016/s0278-6915(99)00021-6 (1999).
- 351 Thapliyal, R., Naresh, K. N., Rao, K. V. & Maru, G. B. Inhibition of nitrosodiethylamine-induced hepatocarcinogenesis by dietary turmeric in rats. *Toxicol Lett* **139**, 45-54, doi:10.1016/s0378-4274(02)00440-x (2003).
- 352 Mohr, U. & Hilfrich, J. Brief communication: effect of a single dose of N-diethylnitrosamine on the rat kidney. *J Natl Cancer Inst* **49**, 1729-1731, doi:10.1093/jnci/49.6.1729 (1972).
- 353 Bose, P. *et al.* Quinazolinone derivative BNUA-3 ameliorated [NDEA+2-AAF]-induced liver carcinogenesis in SD rats by modulating AhR-CYP1B1-Nrf2-Keap1 pathway. *Clin Exp Pharmacol Physiol* **47**, 143-157, doi:10.1111/1440-1681.13184 (2020).
- 354 Giri, R. K., Baral, R. N. & Das, B. R. Induction of lung carcinogenesis in AKR-mice by N-nitrosodiethylamine/phenobarbitone, associated with high expression of c-myc and c-jun oncoproteins. *Cancer Lett* **112**, 57-63, doi:10.1016/S0304-3835(96)04545-4 (1997).
- 355 Gupta, P., Bansal, M. P. & Koul, A. Spectroscopic characterization of lycopene extract from *Lycopersicum esculentum* (Tomato) and its evaluation as a chemopreventive agent against experimental hepatocarcinogenesis in mice. *Phytother Res* **27**, 448-456, doi:10.1002/ptr.4741 (2013).
- 356 Bharati, S., Rishi, P. & Koul, A. *Azadirachta indica* exhibits chemopreventive action against hepatic cancer: Studies on associated histopathological and ultrastructural changes. *Microsc Res Tech* **75**, 586-595, doi:10.1002/jemt.21095 (2012).
- 357 Gupta, P., Bhatia, N., Bansal, M. P. & Koul, A. Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma. *World J Hepatol* **8**, 1222-1233, doi:10.4254/wjh.v8.i29.1222 (2016).
- 358 Hadjiolov, D., Frank, N., Moog, C. & Spirov, K. Proline dithiocarbamate inhibits N-nitrosodiethylamine induced liver carcinogenesis. *J Cancer Res Clin Oncol* **118**, 401-404, doi:10.1007/BF01629420 (1992).
- 359 Kumar, K. B. *et al.* Inhibition of chemically induced carcinogenesis by drugs used in homeopathic medicine. *Asian Pac J Cancer Prev* **8**, 98-102 (2007).
- 360 Hussein, R. H. & Khalifa, F. K. The protective role of ellagitannins flavonoids pretreatment against N-nitrosodiethylamine induced-hepatocellular carcinoma. *Saudi J Biol Sci* **21**, 589-596, doi:10.1016/j.sjbs.2014.03.004 (2014).

- 361 Ishii, N. *et al.* Ascorbic acid prevents N-nitrosodiethylamine-induced hepatic injury and hepatocarcinogenesis in Akr1a-knockout mice. *Toxicol Lett* **333**, 192-201, doi:10.1016/j.toxlet.2020.08.005 (2020).
- 362 Ishikawa, H. *et al.* Bone marrow engraftment in a rodent model of chemical carcinogenesis but no role in the histogenesis of hepatocellular carcinoma. *Gut* **53**, 884-889, doi:10.1136/gut.2003.026047 (2004).
- 363 Jagwani, S., Jalalpure, S., Dhamecha, D., Jadhav, K. & Bohara, R. Pharmacokinetic and Pharmacodynamic Evaluation of Resveratrol Loaded Cationic Liposomes for Targeting Hepatocellular Carcinoma. *ACS Biomater Sci Eng* **6**, 4969-4984, doi:10.1021/acsbiomaterials.0c00429 (2020).
- 364 Wu, F. Q. *et al.* ADRB2 signaling promotes HCC progression and sorafenib resistance by inhibiting autophagic degradation of HIF1alpha. *J Hepatol* **65**, 314-324, doi:10.1016/j.jhep.2016.04.019 (2016).
- 365 Jeyabal, P. V., Syed, M. B., Venkataraman, M., Sambandham, J. K. & Sakthisekaran, D. Apigenin inhibits oxidative stress-induced macromolecular damage in N-nitrosodiethylamine (NDEA)-induced hepatocellular carcinogenesis in Wistar albino rats. *Mol Carcinog* **44**, 11-20, doi:10.1002/mc.20115 (2005).
- 366 Krishnan, P. *et al.* Citral inhibits N-nitrosodiethylamine-induced hepatocellular carcinoma via modulation of antioxidants and xenobiotic-metabolizing enzymes. *Environ Toxicol* **35**, 971-981, doi:10.1002/tox.22933 (2020).
- 367 Kujawska, M. *et al.* Protective Effect of Morus alba Leaf Extract on N-Nitrosodiethylamine-induced Hepatocarcinogenesis in Rats. *In Vivo* **30**, 807-812, doi:10.21873/in vivo.10998 (2016).
- 368 Kujawska, M. *et al.* Protective effect of yellow tea extract on N-nitrosodiethylamine-induced liver carcinogenesis. *Pharm Biol* **54**, 1891-1900, doi:10.3109/13880209.2015.1137600 (2016).
- 369 Kumar, P. *et al.* Poly(lactic-co-glycolic acid)-loaded nanoparticles of betulinic acid for improved treatment of hepatic cancer: characterization, in vitro and in vivo evaluations. *Int J Nanomedicine* **13**, 975-990, doi:10.2147/IJN.S157391 (2018).
- 370 Liao, S. *et al.* Chemoprevention of elite tea variety CFT-1 rich in EGCG against chemically induced liver cancer in rats. *Food Sci Nutr* **7**, 2647-2665, doi:10.1002/fsn3.1121 (2019).
- 371 Bercu, J. P. *et al.* Use of less-than-lifetime (LTL) durational limits for nitrosamines: Case study of N-Nitrosodiethylamine (NDEA). *Regul Toxicol Pharmacol* **123**, 104926, doi:10.1016/j.yrtph.2021.104926 (2021).
- 372 Habs, M. & Schmahl, D. Synergistic effects of N-nitroso compounds in experimental long-term carcinogenesis studies. *Oncology* **37**, 259-265, doi:10.1159/000225448 (1980).
- 373 Nixon, J. E., Sinnhuber, R. O., Lee, D. J., Landers, M. K. & Harr, J. R. Effect of cyclopropenoid compounds on the carcinogenic activity of diethylnitrosamine and aflatoxin B1 in rats. *J Natl Cancer Inst* **53**, 453-458, doi:10.1093/jnci/53.2.453 (1974).
- 374 Lijinsky, W., Reuber, M. D. & Riggs, C. W. Carcinogenesis by combinations of N-nitroso compounds in rats. *Food Chem Toxicol* **21**, 601-605, doi:10.1016/0278-6915(83)90147-3 (1983).
- 375 Dakshayani, K. B., Subramanian, P., Manivasagam, T. & Mohamed Essa, M. Metabolic normalization of alpha-ketoglutarate against N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. *Fundam Clin Pharmacol* **20**, 477-480, doi:10.1111/j.1472-8206.2006.00422.x (2006).
- 376 Schmahl, D. & Scherf, H. R. Carcinogenic activity of N-nitrosodiethylamine in snakes (Python reticulatus, Schneider). *IARC Sci Publ*, 677-682 (1984).
- 377 Schmahl, D. & Scherf, H. R. Carcinogenic activity of N-nitrosodiethylamine in snakes. *Naturwissenschaften* **70**, 94-95, doi:10.1007/BF00365511 (1983).

- 378 Thiagarajah, A. & Grizzle, J. M. Pathology of diethylnitrosamine toxicity in the fish *Rivulus marmoratus*. *J Environ Pathol Toxicol Oncol* **6**, 219-231 (1985).
- 379 Thiagarajah, A. & Grizzle, J. M. Diethylnitrosamine-induced pancreatic neoplasms in the fish *Rivulus ocellatus marmoratus*. *J Natl Cancer Inst* **77**, 141-147 (1986).
- 380 Gray, R., Peto, R., Brantom, P. & Grasso, P. Chronic nitrosamine ingestion in 1040 rodents: the effect of the choice of nitrosamine, the species studied, and the age of starting exposure. *Cancer Res* **51**, 6470-6491 (1991).
- 381 McCann, J., Spingarn, N. E., Kabori, J. & Ames, B. N. Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc Natl Acad Sci U S A* **72**, 979-983 (1975).
- 382 Rastogi, S., Dogra, R. K., Khanna, S. K. & Das, M. Skin tumorigenic potential of aflatoxin B1 in mice. *Food Chem Toxicol* **44**, 670-677, doi:10.1016/j.fct.2005.09.008 (2006).
- 383 Aiub, C. A., Pinto, L. F. & Felzenszwalb, I. N-Nitrosodiethylamine mutagenicity at low concentrations. *Toxicol Lett* **145**, 36-45, doi:10.1016/s0378-4274(03)00263-7 (2003).
- 384 Pegg, A. E. Formation and subsequent repair of alkylation lesions in tissues of rodents treated with nitrosamines. *Arch Toxicol Suppl* **3**, 55-68, doi:10.1007/978-3-642-67389-4_5 (1980).
- 385 Singer, B. In vivo formation and persistence of modified nucleosides resulting from alkylating agents. *Environ Health Perspect* **62**, 41-48, doi:10.1289/ehp.856241 (1985).
- 386 Magee, P. N. & Lee, K. Y. Cellular injury and carcinogenesis. Alkylation of ribonucleic acid of rat liver by diethylnitrosamine and n-butylmethylnitrosamine in vivo. *Biochem J* **91**, 35-42, doi:10.1042/bj0910035 (1964).
- 387 Bartsch, H., Malaveille, C. & Montesano, R. In vitro metabolism and microsome-mediated mutagenicity of dialkyl nitrosamines in rat, hamster, and mouse tissues. *Cancer Res* **35**, 644-651 (1975).
- 388 Fujita, K. & Kamataki, T. Predicting the mutagenicity of tobacco-related N-nitrosamines in humans using 11 strains of *Salmonella typhimurium* YG7108, each coexpressing a form of human cytochrome P450 along with NADPH-cytochrome P450 reductase. *Environ Mol Mutagen* **38**, 339-346, doi:10.1002/em.10036 (2001).
- 389 Frank, N., Wiessler, M. & Hadjiolov, D. Influence of disulfiram on the metabolism of N-nitrosodiethylamine. *IARC Sci Publ*, 519-523 (1984).
- 390 Pegg, A. E. Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest* **2**, 223-231, doi:10.3109/07357908409104376 (1984).
- 391 Singer, B. Alkylation of the O6 of guanine is only one of many chemical events that may initiate carcinogenesis. *Cancer Invest* **2**, 233-238, doi:10.3109/07357908409104377 (1984).
- 392 Dyroff, M. C., Richardson, F. C., Popp, J. A., Bedell, M. A. & Swenberg, J. A. Correlation of O4-ethyldeoxythymidine accumulation, hepatic initiation and hepatocellular carcinoma induction in rats continuously administered diethylnitrosamine. *Carcinogenesis* **7**, 241-246, doi:10.1093/carcin/7.2.241 (1986).
- 393 Camus, A. M. *et al.* High variability of nitrosamine metabolism among individuals: role of cytochromes P450 2A6 and 2E1 in the dealkylation of N-nitrosodimethylamine and N-nitrosodiethylamine in mice and humans. *Mol Carcinog* **7**, 268-275, doi:10.1002/mc.2940070410 (1993).
- 394 Song, B. J., Gelboin, H. V., Park, S. S., Yang, C. S. & Gonzalez, F. J. Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450s. Transcriptional and post-transcriptional regulation of the rat enzyme. *J Biol Chem* **261**, 16689-16697 (1986).
- 395 Crespi, C. L. *et al.* Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis* **11**, 1293-1300, doi:10.1093/carcin/11.8.1293 (1990).

- 396 Scherer, E., Van Den Berg, T., Vermeulen, E., Winterwerp, H. H. & Den Engelse, L. Immunocytochemical analysis of O6-alkylguanine shows tissue specific formation in and removal from esophageal and liver DNA in rats treated with methylbenzylNitrosamine, dimethylNitrosamine, diethylNitrosamine and ethylNitrosourea. *Cancer Lett* **46**, 21-29, doi:10.1016/0304-3835(89)90210-3 (1989).
- 397 Aitio, A., Aitio, M. L., Camus, A. M., Cardis, E. & Bartsch, H. Cytochrome P-450 isozyme pattern is related to individual susceptibility to diethylNitrosamine-induced liver cancer in rats. *Jpn J Cancer Res* **82**, 146-156 (1991).
- 398 Versteeg, G. A., van de Nes, P. S., Bredenbeek, P. J. & Spaan, W. J. The coronavirus spike protein induces endoplasmic reticulum stress and upregulation of intracellular chemokine mRNA concentrations. *J Virol* **81**, 10981-10990, doi:10.1128/JVI.01033-07 (2007).
- 399 McCann, J., Choi, E., Yamasaki, E. & Ames, B. N. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. *Proc Natl Acad Sci U S A* **72**, 5135-5139, doi:10.1073/pnas.72.12.5135 (1975).
- 400 Zeiger, E. *et al.* Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. *Environ Mutagen* **9 Suppl 9**, 1-109 (1987).
- 401 Aiub, C. A., Pinto, L. F. & Felzenszwalb, I. Standardization of conditions for the metabolic activation of N-nitrosodiethylamine in mutagenicity tests. *Genet Mol Res* **3**, 264-272 (2004).
- 402 Maron, D. M. & Ames, B. N. Revised methods for the Salmonella mutagenicity test. *Mutat Res* **113**, 173-215, doi:10.1016/0165-1161(83)90010-9 (1983).
- 403 Quillardet, P. & Hofnung, M. The SOS Chromotest, a colorimetric bacterial assay for genotoxins: procedures. *Mutat Res* **147**, 65-78, doi:10.1016/0165-1161(85)90020-2 (1985).
- 404 Ehrenberg, L. Covalent binding of genotoxic agents to proteins and nucleic acids. *IARC Sci Publ*, 107-114 (1984).
- 405 Ali, F., Rahul, Naz, F., Jyoti, S. & Siddique, Y. H. Protective effect of apigenin against N-nitrosodiethylamine (NDEA)-induced hepatotoxicity in albino rats. *Mutat Res Genet Toxicol Environ Mutagen* **767**, 13-20, doi:10.1016/j.mrgentox.2014.04.006 (2014).
- 406 Aiub, C. A. *et al.* N-nitrosodiethylamine genotoxicity evaluation: a cytochrome P450 induction study in rat hepatocytes. *Genet Mol Res* **10**, 2340-2348, doi:10.4238/2011.October.5.4 (2011).
- 407 Kleinsasser, N. H., Wallner, B. C., Wagner, C., Kastenbauer, E. R. & Harreus, U. A. DNA repair capacity in lymphocytes of nasopharyngeal cancer patients. *Eur Arch Otorhinolaryngol* **262**, 561-566, doi:10.1007/s00405-004-0872-3 (2005).
- 408 Lee, B. H., Bertram, B., Schmezer, P., Frank, N. & Wiessler, M. Sugar-linked dithiocarbamates as modulators of metabolic and genotoxic properties of N-nitroso compounds. *J Med Chem* **37**, 3154-3162, doi:10.1021/jm00045a021 (1994).
- 409 Kleinsasser, N. H., Kastenbauer, E. R., Weissacher, H., Muenzenrieder, R. K. & Harreus, U. A. Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. *Environ Mol Mutagen* **35**, 9-12, doi:10.1002/(sici)1098-2280(2000)35:1<9::aid-em2>3.0.co;2-1 (2000).
- 410 Bharati, S., Rishi, P. & Koul, A. Azadirachta indica modulates electrical properties and type of cell death in NDEA-induced hepatic tumors. *Cell Biochem Biophys* **70**, 383-390, doi:10.1007/s12013-014-9923-6 (2014).
- 411 Montesano, R. & Bartsch, H. Mutagenic and carcinogenic N-nitroso compounds: possible environmental hazards. *Mutat Res* **32**, 179-228, doi:10.1016/0165-1110(76)90001-4 (1976).
- 412 Pegg, A. E. & Byers, T. L. Repair of DNA containing O6-alkylguanine. *FASEB J* **6**, 2302-2310, doi:10.1096/fasebj.6.6.1544541 (1992).

- 413 Aiub, C. A., Pinto, L. F. & Felzenszwalb, I. DNA-repair genes and vitamin E in the prevention of N-nitrosodiethylamine mutagenicity. *Cell Biol Toxicol* **25**, 393-402, doi:10.1007/s10565-008-9093-7 (2009).
- 414 Josephy, P. D., Lord, H. L. & Snieckus, V. A. Dimethylnitrosamine genotoxicity: does N-acetyltransferase activity play a role? *Carcinogenesis* **15**, 479-482, doi:10.1093/carcin/15.3.479 (1994).
- 415 Yamazaki, H. *et al.* Participation of rat liver cytochrome P450 2E1 in the activation of N-nitrosodimethylamine and N-nitrosodiethylamine to products genotoxic in an acetyltransferase-overexpressing Salmonella typhimurium strain (NM2009). *Carcinogenesis* **13**, 979-985, doi:10.1093/carcin/13.6.979 (1992).
- 416 Natarajan, A. T., Bates, A. D., Van Buul, P. P., Meijers, M. & De Vogel, N. Cytogenetic effects of mutagens/carcinogens after activation in a microsomal system in vitro I. Induction of chromosome aberrations and sister chromatid exchanges by diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) in CHO cells in the presence of rat-liver microsomes. *Mutat Res* **37**, 83-90, doi:10.1016/0027-5107(76)90057-9 (1976).
- 417 Yamashita, Y., Sumi, N., Arimoto, S. & Hayatsu, H. Synergistic action of N-nitrosodialkylamines and near-UV in the induction of chromosome aberrations in Chinese hamster lung fibroblasts in vitro. *Mutat Res* **348**, 163-168, doi:10.1016/0165-7992(95)90004-7 (1995).
- 418 You, M. *et al.* Mutagenesis of the K-ras protooncogene in mouse lung tumors induced by N-ethyl-N-nitrosourea or N-nitrosodiethylamine. *Carcinogenesis* **13**, 1583-1586, doi:10.1093/carcin/13.9.1583 (1992).
- 419 Jin, G. *et al.* Mutagenic Activity of N-Nitrosodiethylamine in Cell Lines Expressing Human CYP2E1-Adequacy of Dimethylsulfoxide as Solvent. *Environ Mol Mutagen* **60**, 214-226, doi:10.1002/em.22264 (2019).
- 420 Ajiboye, T. O. *et al.* Bridelia ferruginea promotes reactive oxygen species detoxification in N-nitrosodiethylamine-treated rats. *J Diet Suppl* **10**, 210-228, doi:10.3109/19390211.2013.822451 (2013).
- 421 Ampy, F. R. & Williams, A. O. Dimethylnitrosamine metabolism: I. In vitro activation of dimethylnitrosamine to mutagenic substance(s) by hepatic and renal tissues from three inbred strains of mice. *Life Sci* **39**, 923-930, doi:10.1016/0024-3205(86)90374-7 (1986).
- 422 Negrini, S., Gorgoulis, V. G. & Halazonetis, T. D. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* **11**, 220-228, doi:10.1038/nrm2858 (2010).
- 423 von Hofe, E. & Kleihues, P. Comparative studies on hepatic DNA alkylation in rats by N-nitrosomethylethylamine and N-nitrosodimethylamine plus N-nitrosodiethylamine. *J Cancer Res Clin Oncol* **112**, 205-209, doi:10.1007/BF00395913 (1986).
- 424 Khare, S. P., Sharma, A., Deodhar, K. K. & Gupta, S. Overexpression of histone variant H2A.1 and cellular transformation are related in N-nitrosodiethylamine-induced sequential hepatocarcinogenesis. *Exp Biol Med (Maywood)* **236**, 30-35, doi:10.1258/ebm.2010.010140 (2011).
- 425 Bansal, A. K., Trivedi, R., Soni, G. L. & Bhatnagar, D. Hepatic and renal oxidative stress in acute toxicity of N-nitrosodiethylamine in rats. *Indian J Exp Biol* **38**, 916-920 (2000).
- 426 Bansal, A. K., Bansal, M., Soni, G. & Bhatnagar, D. Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem Biol Interact* **156**, 101-111, doi:10.1016/j.cbi.2005.08.001 (2005).
- 427 Singh, J. P., Selvendiran, K., Banu, S. M., Padmavathi, R. & Sakthisekaran, D. Protective role of Apigenin on the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in Wistar albino rats. *Phytomedicine* **11**, 309-314, doi:10.1078/0944711041495254 (2004).

- 428 Kujawska, M. *et al.* Antioxidant effect of lycopene-enriched tomato paste on N-nitrosodiethylamine-induced oxidative stress in rats. *J Physiol Biochem* **70**, 981-990, doi:10.1007/s13105-014-0367-7 (2014).
- 429 Guengerich, F. P., Kim, D. H. & Iwasaki, M. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4**, 168-179, doi:10.1021/tx00020a008 (1991).
- 430 de Zwart, L. L., Meerman, J. H., Commandeur, J. N. & Vermeulen, N. P. Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med* **26**, 202-226, doi:10.1016/s0891-5849(98)00196-8 (1999).
- 431 Bartsch, H., Hietanen, E. & Malaveille, C. Carcinogenic nitrosamines: free radical aspects of their action. *Free Radic Biol Med* **7**, 637-644 (1989).
- 432 Ali, F. *et al.* Protective Role of Curcumin against N-Nitrosodiethylamine (NDEA)-Induced Toxicity in Rats. *Sci Pharm* **84**, 361-377, doi:10.3797/scipharm.1506-06 (2016).
- 433 El Mesallamy, H. O., Metwally, N. S., Soliman, M. S., Ahmed, K. A. & Abdel Moaty, M. M. The chemopreventive effect of Ginkgo biloba and Silybum marianum extracts on hepatocarcinogenesis in rats. *Cancer Cell Int* **11**, 38, doi:10.1186/1475-2867-11-38 (2011).
- 434 Chuang, S. E., Cheng, A. L., Lin, J. K. & Kuo, M. L. Inhibition by curcumin of diethylnitrosamine-induced hepatic hyperplasia, inflammation, cellular gene products and cell-cycle-related proteins in rats. *Food Chem Toxicol* **38**, 991-995, doi:10.1016/s0278-6915(00)00101-0 (2000).
- 435 Bhatia, N., Gupta, P., Singh, B. & Koul, A. Lycopene Enriched Tomato Extract Inhibits Hypoxia, Angiogenesis, and Metastatic Markers in early Stage N-Nitrosodiethylamine Induced Hepatocellular Carcinoma. *Nutr Cancer* **67**, 1268-1275, doi:10.1080/01635581.2015.1087040 (2015).
- 436 Husain, H., Latief, U. & Ahmad, R. Pomegranate action in curbing the incidence of liver injury triggered by Diethylnitrosamine by declining oxidative stress via Nrf2 and NFkappaB regulation. *Sci Rep* **8**, 8606, doi:10.1038/s41598-018-26611-1 (2018).
- 437 Shaban, N. Z., El-Kersh, M. A., El-Rashidy, F. H. & Habashy, N. H. Protective role of Punica granatum (pomegranate) peel and seed oil extracts on diethylnitrosamine and phenobarbital-induced hepatic injury in male rats. *Food Chem* **141**, 1587-1596, doi:10.1016/j.foodchem.2013.04.134 (2013).
- 438 Balkwill, F., Charles, K. A. & Mantovani, A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7**, 211-217, doi:S1535-6108(05)00066-8 [pii] 10.1016/j.ccr.2005.02.013 (2005).
- 439 Fishbein, A., Hammock, B. D., Serhan, C. N. & Panigrahy, D. Carcinogenesis: Failure of resolution of inflammation? *Pharmacol Ther*, 107670, doi:10.1016/j.pharmthera.2020.107670 (2020).
- 440 Ahmed, H. H., Khalil, W. K. & Hamza, A. H. Molecular mechanisms of nano-selenium in mitigating hepatocellular carcinoma induced by N-nitrosodiethylamine (NDEA) in rats. *Toxicol Mech Methods* **24**, 593-602, doi:10.3109/15376516.2014.956912 (2014).
- 441 Chang, Q., Daly, L. & Bromberg, J. The IL-6 feed-forward loop: a driver of tumorigenesis. *Semin Immunol* **26**, 48-53, doi:10.1016/j.smim.2014.01.007 (2014).
- 442 Bao, X. *et al.* Sphingosine 1-phosphate promotes the proliferation of olfactory ensheathing cells through YAP signaling and participates in the formation of olfactory nerve layer. *Glia*, doi:10.1002/glia.23803 (2020).
- 443 Wolf, T., Niehaus-Rolf, C. & Luepke, N. P. Investigating genotoxic and hematotoxic effects of N-nitrosodimethylamine, N-nitrosodiethylamine and N-nitrosodiethanolamine in the hen's egg-micronucleus test (HET-MN). *Food Chem Toxicol* **41**, 561-573 (2003).

- 444 Akira, S., Taga, T. & Kishimoto, T. Interleukin-6 in biology and medicine. *Adv Immunol* **54**, 1-78, doi:10.1016/s0065-2776(08)60532-5 (1993).
- 445 Huang, M., Wang, X. Q., Wang, Z. H., Zhou, C. N. & Wu, M. The studies of malignant transformation of human epithelial cells induced by diethyl-nitrosamine (NDEA) in vitro. *Sci Sin B* **29**, 1186-1191 (1986).
- 446 DiPaolo, J. A., Nelson, R. L. & Donovan, P. J. In vitro transformation of Syrian hamster embryo cells by diverse chemical carcinogens. *Nature* **235**, 278-280, doi:10.1038/235278a0 (1972).
- 447 Evans, C. H. & DiPaolo, J. A. Neoplastic transformation of guinea pig fetal cells in culture induced by chemical carcinogens. *Cancer Res* **35**, 1035-1044 (1975).
- 448 Huang, M., Wang, Z. H., Wang, X. Q. & Wu, M. Malignant transformation of human fetal lung fibroblasts induced by nitrosamine compounds in vitro. *Sci Sin B* **29**, 1192-1200 (1986).
- 449 Emura, M., Mohr, U., Kakunaga, T. & Hilfrich, J. Growth inhibition and transformation of a human fetal tracheal epithelial cell line by long-term exposure to diethylnitrosamine. *Carcinogenesis* **6**, 1079-1085, doi:10.1093/carcin/6.8.1079 (1985).
- 450 Rajewsky, M. F. [Cell proliferation in normal and malignant tissues: H3-thymidine incorporation in vitro under standard conditions]. *Biophysik* **3**, 65-93, doi:10.1007/BF01191058 (1966).
- 451 Rajewsky, M. F. Proliferative parameters of mammalian cell systems and their role in tumor growth and carcinogenesis. *Z Krebsforsch Klin Onkol Cancer Res Clin Oncol* **78**, 12-30, doi:10.1007/BF00284309 (1972).
- 452 Deal, F. H., Richardson, F. C. & Swenberg, J. A. Dose response of hepatocyte replication in rats following continuous exposure to diethylnitrosamine. *Cancer Res* **49**, 6985-6988 (1989).
- 453 Lim, I. K. Spectrum of molecular changes during hepatocarcinogenesis induced by DEN and other chemicals in Fisher 344 male rats [Mechanisms of Ageing and Development 123 (2002) 1665-1680]. *Mech Ageing Dev* **124**, 697-708, doi:10.1016/s0047-6374(03)00010-1 (2003).
- 454 Itoh, Y. & Nagase, H. Matrix metalloproteinases in cancer. *Essays Biochem* **38**, 21-36, doi:10.1042/bse0380021 (2002).
- 455 Goto, Y. *et al.* Mutagenicities of N-nitrosodimethylamine and N-nitrosodiethylamine in *Drosophila* and their relationship to the levels of O-alkyl adducts in DNA. *Mutat Res* **425**, 125-134, doi:10.1016/s0027-5107(99)00032-9 (1999).
- 456 Gorsky, L. D. & Hollenberg, P. F. Metabolism of N-nitrosodimethyl- and N-nitrosodiethylamine by rat hepatocytes: effects of pretreatment with ethanol. *Chem Res Toxicol* **2**, 436-441, doi:10.1021/tx00012a013 (1989).
- 457 Stowers, S. J. *et al.* Detection of activated proto-oncogenes in N-nitrosodiethylamine-induced liver tumors: a comparison between B6C3F1 mice and Fischer 344 rats. *Carcinogenesis* **9**, 271-276, doi:10.1093/carcin/9.2.271 (1988).
- 458 Giri, R. K. & Das, B. R. Differential expression of c-jun and c-myc in N-nitroso diethylamine-induced hepatic oncogenesis in AKR mice. *Cancer Lett* **109**, 121-127, doi:10.1016/s0304-3835(96)04446-1 (1996).
- 459 Berger, M. R., Schmahl, D. & Edler, L. Implications of the carcinogenic hazard of low doses of three hepatocarcinogenic N-nitrosamines. *Jpn J Cancer Res* **81**, 598-606, doi:10.1111/j.1349-7006.1990.tb02615.x (1990).
- 460 Herrold, K. M. & Dunham, L. J. Induction of tumors in the Syrian hamster with diethylnitrosamine (N-nitrosodiethylamine). *Cancer Res* **23**, 773-777 (1963).
- 461 Thomas, C. & Schmahl, D. [Comparative morphological and toxicological studies on the mouse, rat, guinea pig, rabbit and dog for testing the cancerogenic effects and organotropism of diethylnitrosamine]. *Virchows Arch Pathol Anat Physiol Klin Med* **340**, 122-136 (1965).
- 462 Schmahl, D., Habs, M. & Ivankovic, S. Carcinogenesis of N-nitrosodiethylamine (DNA) in chickens and domestic cats. *Int J Cancer* **22**, 552-557, doi:10.1002/ijc.2910220508 (1978).

- 463 Hoch-Ligeti, C., Lobl, L. T. & Arvin, J. M. Effect of Nitrosamine Derivatives on Enzyme
Concentrations in Rat Organs during Carcinogenesis. *Br J Cancer* **18**, 271-284,
doi:10.1038/bjc.1964.32 (1964).
- 464 Hadjiolov, D. Hemangioendothelial sarcomas of the liver in rats induced by diethylnitrosamine.
Neoplasma **19**, 111-114 (1972).
- 465 Thomas, C. & Schmaehl, D. [on the Morphology of Diethylnitrosamine-Induced Liver Tumors in
Mice and Guinea Pigs]. *Z Krebsforsch* **65**, 531-536 (1963).
- 466 Schmaehl, D. & Thomas, C. [Experimental investigation of "syncarcinogenesis". 4. Studies of the
induction of cancer in rats with the simultaneous oral administration of diethylnitrosamine and
4-dimethylaminostilbene]. *Z Krebsforsch* **67**, 135-140 (1965).
- 467 Rapp, H. J., Carleton, J. H., Crisler, C. & Nadel, E. M. Induction of Malignant Tumors in the Rabbit
by Oral Administration of Diethylnitrosamine. *J Natl Cancer Inst* **34**, 453-458 (1965).
- 468 Hirao, K., Matsumura, K., Imagawa, A., Enomoto, Y. & Hosogi, Y. Primary neoplasms in dog liver
induced by diethylnitrosamine. *Cancer Res* **34**, 1870-1882 (1974).
- 469 Schmaehl, D., Osswald, H. & Mohr, U. [Hepatotoxic and cacinogenic effect of diethylnitrosamine
in swine]. *Naturwissenschaften* **54**, 341, doi:10.1007/BF00621459 (1967).
- 470 Graw, J. J., Berg, H. & Schmaehl, D. Carcinogenic and hepatotoxic effects of diethylnitrosamine in
hedgehogs. *J Natl Cancer Inst* **53**, 589, doi:10.1093/jnci/53.2.589 (1974).
- 471 Schmaehl, D., Osswald, H. & Karsten, C. [Liver cancer production by diethylnitrosamine in
budgerigars]. *Naturwissenschaften* **53**, 437, doi:10.1007/BF00654523 (1966).
- 472 Herrold, K. M. Effect of Route of Administration on the Carcinogenic Action of
Diethylnitrosamine (N-Nitrosodiethylamine). *Br J Cancer* **18**, 763-767, doi:10.1038/bjc.1964.88
(1964).
- 473 Herrold, K. M. Induction of Olfactory Neuroepithelial Tumors in Syrian Hamsters by
Diethylnitrosamine. *Cancer* **17**, 114-121, doi:10.1002/1097-0142(196401)17:1<114::aid-
cncr2820170115>3.0.co;2-q (1964).
- 474 Taha, M. M. *et al.* Potential chemoprevention of diethylnitrosamine-initiated and 2-
acetylaminofluorene-promoted hepatocarcinogenesis by zerumbone from the rhizomes of the
subtropical ginger (*Zingiber zerumbet*). *Chem Biol Interact* **186**, 295-305,
doi:10.1016/j.cbi.2010.04.029 (2010).
- 475 Kojiro, M. Histopathology of liver cancers. *Best Pract Res Clin Gastroenterol* **19**, 39-62,
doi:10.1016/j.bpg.2004.10.007 (2005).
- 476 Mirvish, S. S. & Kaufman, L. A study of nitrosamines and S-carboxyl derivatives of cysteine as
lung carcinogens in adult SWR mice. *Int J Cancer* **6**, 69-73, doi:10.1002/ijc.2910060111 (1970).
- 477 Gargus, J. L., Paynter, O. E. & Reese, W. H., Jr. Utilization of newborn mice in the bioassay of
chemical carcinogens. *Toxicol Appl Pharmacol* **15**, 552-559, doi:10.1016/0041-008x(69)90057-x
(1969).
- 478 Khanduja, K. L., Koul, I. B., Gandhi, R. K., Sehgal, S. & Sharma, R. R. Effect of combined deficiency
of fat and vitamin A on N-nitrosodiethylamine-induced lung carcinogenesis in mice. *Cancer Lett*
62, 57-62, doi:10.1016/0304-3835(92)90198-5 (1992).
- 479 Diwan, B. A. & Meier, H. Carcinogenic effects of a single dose of diethylnitrosamine in three
unrelated strains of mice: genetic dependence of the induced tumor types and incidence.
Cancer Lett **1**, 249-253, doi:10.1016/s0304-3835(75)97372-3 (1976).
- 480 Kyriazis, A. P., Koka, M. & Vesselinovitch, S. D. Metastatic rate of liver tumors induced by
diethylnitrosamine in mice. *Cancer Res* **34**, 2881-2886 (1974).
- 481 Hilfrich, J., Schmeltz, I. & Hoffmann, D. Effects of N-nitrosodiethanolamine and 1,1-
diethanolhydrazine in Syrian golden hamsters. *Cancer Lett* **4**, 55-60, doi:10.1016/s0304-
3835(78)93412-2 (1978).

- 482 Hueper, K. *et al.* PET/CT imaging of c-Myc transgenic mice identifies the genotoxic N-nitroso-
diethylamine as carcinogen in a short-term cancer bioassay. *PLoS One* **7**, e30432,
doi:10.1371/journal.pone.0030432 (2012).
- 483 Baker, J. R., Mason, M. M., Yerganian, G., Weisburger, E. K. & Weisburger, J. H. Induction of
tumors of the stomach and esophagus in inbred Chinese hamsters by oral diethylnitrosamine.
Proc Soc Exp Biol Med **146**, 291-293, doi:10.3181/00379727-146-38090 (1974).
- 484 Reznik, G., Mohr, U. & Kmoch, N. Carcinogenic effects of different nitroso-compounds in Chinese
hamsters. I. Dimethylnitrosamine and N-diethylnitrosamine. *Br J Cancer* **33**, 411-418,
doi:10.1038/bjc.1976.66 (1976).
- 485 Reuber, M. D. Carcinomas of the esophagus in rats ingesting diethylnitrosamine. *Eur J Cancer* **11**,
97-99, doi:10.1016/0014-2964(75)90185-1 (1975).
- 486 Urbach, D., Lupien, M., Karagas, M. R. & Moore, J. H. Cancer heterogeneity: origins and
implications for genetic association studies. *Trends Genet* **28**, 538-543,
doi:10.1016/j.tig.2012.07.001 (2012).
- 487 Husemann, Y. *et al.* Systemic spread is an early step in breast cancer. *Cancer Cell* **13**, 58-68,
doi:10.1016/j.ccr.2007.12.003 (2008).
- 488 Braun, S. *et al.* Cytokeratin-positive cells in the bone marrow and survival of patients with stage
I, II, or III breast cancer. *N Engl J Med* **342**, 525-533, doi:10.1056/NEJM200002243420801
(2000).
- 489 Nguyen, D. X., Bos, P. D. & Massague, J. Metastasis: from dissemination to organ-specific
colonization. *Nat Rev Cancer* **9**, 274-284, doi:10.1038/nrc2622 (2009).
- 490 Fidler, I. J. & Kripke, M. L. The challenge of targeting metastasis. *Cancer Metastasis Rev* **34**, 635-
641, doi:10.1007/s10555-015-9586-9 (2015).
- 491 Neophytou, C. M., Kyriakou, T. C. & Papageorgis, P. Mechanisms of Metastatic Tumor Dormancy
and Implications for Cancer Therapy. *Int J Mol Sci* **20**, doi:10.3390/ijms20246158 (2019).
- 492 Recasens, A. & Munoz, L. Targeting Cancer Cell Dormancy. *Trends Pharmacol Sci* **40**, 128-141,
doi:10.1016/j.tips.2018.12.004 (2019).
- 493 Kareva, I. Primary and metastatic tumor dormancy as a result of population heterogeneity. *Biol
Direct* **11**, 37, doi:10.1186/s13062-016-0139-0 (2016).
- 494 Aguirre-Ghiso, J. A. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev
Cancer* **7**, 834-846, doi:10.1038/nrc2256 (2007).
- 495 Retsky, M. W., Demicheli, R., Hrushesky, W. J., Baum, M. & Gukas, I. D. Dormancy and surgery-
driven escape from dormancy help explain some clinical features of breast cancer. *APMIS* **116**,
730-741, doi:10.1111/j.1600-0463.2008.00990.x (2008).
- 496 Indraccolo, S. Insights into the regulation of tumor dormancy by angiogenesis in experimental
tumors. *Adv Exp Med Biol* **734**, 37-52, doi:10.1007/978-1-4614-1445-2_3 (2013).
- 497 Summers, M. A., McDonald, M. M. & Croucher, P. I. Cancer Cell Dormancy in Metastasis. *Cold
Spring Harb Perspect Med* **10**, doi:10.1101/cshperspect.a037556 (2020).
- 498 Goodison, S. *et al.* Prolonged dormancy and site-specific growth potential of cancer cells
spontaneously disseminated from nonmetastatic breast tumors as revealed by labeling with
green fluorescent protein. *Clin Cancer Res* **9**, 3808-3814 (2003).
- 499 Naumov, G. N., Akslen, L. A. & Folkman, J. Role of angiogenesis in human tumor dormancy:
animal models of the angiogenic switch. *Cell Cycle* **5**, 1779-1787 (2006).
- 500 Krall, J. A. *et al.* The systemic response to surgery triggers the outgrowth of distant immune-
controlled tumors in mouse models of dormancy. *Sci Transl Med* **10**,
doi:10.1126/scitranslmed.aan3464 (2018).
- 501 Fisher, B. & Fisher, E. R. Experimental evidence in support of the dormant tumor cell. *Science*
130, 918-919 (1959).

- 502 Folkman, J. in *Harrison's Textbook of Internal Medicine* (eds E. Braunwald *et al.*) 517-530 (McGraw-Hill, 2001).
- 503 Harach, H. R., Franssila, K. O. & Wasenius, V. M. Occult papillary carcinoma of the thyroid. A "normal" finding in Finland. A systematic autopsy study. *Cancer* **56**, 531-538 (1985).
- 504 Naumov, G. N., MacDonald, I. C., Chambers, A. F. & Groom, A. C. Solitary cancer cells as a possible source of tumour dormancy? *Semin Cancer Biol* **11**, 271-276, doi:10.1006/scbi.2001.0382 S1044-579X(01)90382-7 [pii] (2001).
- 505 Naumov, G. N. *et al.* Persistence of solitary mammary carcinoma cells in a secondary site: a possible contributor to dormancy. *Cancer Res* **62**, 2162-2168 (2002).
- 506 Naumov, G. N., Folkman, J. & Straume, O. Tumor dormancy due to failure of angiogenesis: role of the microenvironment. *Clin Exp Metastasis* **26**, 51-60, doi:10.1007/s10585-008-9176-0 (2009).
- 507 Naumov, G. N. *et al.* A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. *J Natl Cancer Inst* **98**, 316-325, doi:98/5/316 [pii] 10.1093/jnci/djj068 (2006).
- 508 Almog, N. Molecular mechanisms underlying tumor dormancy. *Cancer Lett* **294**, 139-146, doi:S0304-3835(10)00141-2 [pii] 10.1016/j.canlet.2010.03.004 (2010).
- 509 Holmgren, L., O'Reilly, M. S. & Folkman, J. Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Med.* **1**, 149-153 (1995).
- 510 Almog, N. *et al.* Prolonged dormancy of human liposarcoma is associated with impaired tumor angiogenesis. *FASEB J* **20**, 947-949, doi:fj.05-3946fje [pii] 10.1096/fj.05-3946fje (2006).
- 511 Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364 (1996).
- 512 Zhu, X. *et al.* FBX8 promotes metastatic dormancy of colorectal cancer in liver. *Cell Death Dis* **11**, 622, doi:10.1038/s41419-020-02870-7 (2020).
- 513 Lin, W. C., Rajbhandari, N. & Wagner, K. U. Cancer cell dormancy in novel mouse models for reversible pancreatic cancer: a lingering challenge in the development of targeted therapies. *Cancer Res* **74**, 2138-2143, doi:10.1158/0008-5472.CAN-13-3437 (2014).
- 514 Dioufa, N., Clark, A. M., Ma, B., Beckwitt, C. H. & Wells, A. Bi-directional exosome-driven intercommunication between the hepatic niche and cancer cells. *Mol Cancer* **16**, 172, doi:10.1186/s12943-017-0740-6 (2017).
- 515 Qiu, Y. *et al.* Biomaterial 3D collagen I gel culture model: A novel approach to investigate tumorigenesis and dormancy of bladder cancer cells induced by tumor microenvironment. *Biomaterials* **256**, 120217, doi:10.1016/j.biomaterials.2020.120217 (2020).
- 516 Pavan Grandhi, T. S., Potta, T., Nitiyanandan, R., Deshpande, I. & Rege, K. Chemomechanically engineered 3D organotypic platforms of bladder cancer dormancy and reactivation. *Biomaterials* **142**, 171-185, doi:10.1016/j.biomaterials.2017.07.008 (2017).
- 517 Fujita, T. *et al.* Identification and Characterization of CXCR4-Positive Gastric Cancer Stem Cells. *PLoS One* **10**, e0130808, doi:10.1371/journal.pone.0130808 (2015).
- 518 Nakayama, T., Ling, Z. Q., Mukaisho, K., Hattori, T. & Sugihara, H. Lineage analysis of early and advanced tubular adenocarcinomas of the stomach: continuous or discontinuous? *BMC Cancer* **10**, 311, doi:10.1186/1471-2407-10-311 (2010).
- 519 Essers, M. A. *et al.* IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908, doi:10.1038/nature07815 (2009).

- 520 Khoo, W. H. *et al.* A niche-dependent myeloid transcriptome signature defines dormant
myeloma cells. *Blood* **134**, 30-43, doi:10.1182/blood.2018880930 (2019).
- 521 Albregues, J. *et al.* Neutrophil extracellular traps produced during inflammation awaken
dormant cancer cells in mice. *Science* **361**, doi:10.1126/science.aao4227 (2018).
- 522 Coussens, L. M., Zitvogel, L. & Palucka, A. K. Neutralizing tumor-promoting chronic
inflammation: a magic bullet? *Science* **339**, 286-291, doi:10.1126/science.1232227 (2013).
- 523 Greene, E. R., Huang, S., Serhan, C. N. & Panigrahy, D. Regulation of inflammation in cancer by
eicosanoids. *Prostaglandins Other Lipid Mediat* **96**, 27-36,
doi:10.1016/j.prostaglandins.2011.08.004 (2011).
- 524 Park, S. Y. & Nam, J. S. The force awakens: metastatic dormant cancer cells. *Exp Mol Med* **52**,
569-581, doi:10.1038/s12276-020-0423-z (2020).
- 525 Kiraly, O., Gong, G., Olipitz, W., Muthupalani, S. & Engelward, B. P. Inflammation-induced cell
proliferation potentiates DNA damage-induced mutations in vivo. *PLoS Genet* **11**, e1004901,
doi:10.1371/journal.pgen.1004901 (2015).
- 526 Dillekas, H., Rogers, M. S. & Straume, O. Are 90% of deaths from cancer caused by metastases?
Cancer Med **8**, 5574-5576, doi:10.1002/cam4.2474 (2019).
- 527 El Rayes, T. *et al.* Lung inflammation promotes metastasis through neutrophil protease-
mediated degradation of Tsp-1. *Proc Natl Acad Sci U S A* **112**, 16000-16005,
doi:10.1073/pnas.1507294112 (2015).
- 528 Stanton, M. F. Diethylnitrosamine-Induced Hepatic Degeneration and Neoplasia in the Aquarium
Fish, Brachydanio Rerio. *J Natl Cancer Inst* **34**, 117-130, doi:10.1093/jnci/34.1.117 (1965).
- 529 Seehawer, M. *et al.* Necroptosis microenvironment directs lineage commitment in liver cancer.
Nature **562**, 69-75, doi:10.1038/s41586-018-0519-y (2018).
- 530 Yu, L. X., Ling, Y. & Wang, H. Y. Role of nonresolving inflammation in hepatocellular carcinoma
development and progression. *NPJ Precis Oncol* **2**, 6, doi:10.1038/s41698-018-0048-z (2018).
- 531 Greten, F. R. *et al.* IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-
associated cancer. *Cell* **118**, 285-296 (2004).
- 532 Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F. & Loguercio, C. Chronic inflammation and
oxidative stress in human carcinogenesis. *Int J Cancer* **121**, 2381-2386, doi:10.1002/ijc.23192
(2007).
- 533 Dairkee, S. H., Luciani-Torres, G., Moore, D. H., Jaffee, I. M. & Goodson, W. H., 3rd. A Ternary
Mixture of Common Chemicals Perturbs Benign Human Breast Epithelial Cells More Than the
Same Chemicals Do Individually. *Toxicol Sci* **165**, 131-144, doi:10.1093/toxsci/kfy126 (2018).
- 534 Ananth, A. A. *et al.* Surgical Stress Abrogates Pre-Existing Protective T Cell Mediated Anti-Tumor
Immunity Leading to Postoperative Cancer Recurrence. *PLoS One* **11**, e0155947,
doi:10.1371/journal.pone.0155947 (2016).
- 535 Lee, J. W. *et al.* Surgical stress promotes tumor growth in ovarian carcinoma. *Clin Cancer Res* **15**,
2695-2702, doi:10.1158/1078-0432.CCR-08-2966 (2009).
- 536 Antonio, N. *et al.* The wound inflammatory response exacerbates growth of pre-neoplastic cells
and progression to cancer. *EMBO J* **34**, 2219-2236, doi:10.15252/embj.201490147 (2015).
- 537 Mathenge, E. G. *et al.* Core needle biopsy of breast cancer tumors increases distant metastases
in a mouse model. *Neoplasia* **16**, 950-960, doi:10.1016/j.neo.2014.09.004 (2014).
- 538 Alieva, M. *et al.* Preventing inflammation inhibits biopsy-mediated changes in tumor cell
behavior. *Sci Rep* **7**, 7529, doi:10.1038/s41598-017-07660-4 (2017).
- 539 Hobson, J. *et al.* Acute inflammation induced by the biopsy of mouse mammary tumors
promotes the development of metastasis. *Breast Cancer Res Treat* **139**, 391-401,
doi:10.1007/s10549-013-2575-1 (2013).

- 540 Retsky, M., Demicheli, R., Hrushesky, W., Baum, M. & Gukas, I. Surgery triggers outgrowth of
latent distant disease in breast cancer: an inconvenient truth? *Cancers (Basel)* **2**, 305-337,
doi:10.3390/cancers2020305 (2010).
- 541 Revesz, L. Effect of tumour cells killed by x-rays upon the growth of admixed viable cells. *Nature*
178, 1391-1392 (1956).
- 542 Shakhari, G. & Ben-Eliyahu, S. Potential prophylactic measures against postoperative
immunosuppression: could they reduce recurrence rates in oncological patients? *Ann Surg Oncol*
10, 972-992 (2003).
- 543 Forget, P., Simonet, O. & De Kock, M. Cancer surgery induces inflammation, immunosuppression
and neo-angiogenesis, but is it influenced by analgesics? *F1000Res* **2**, 102,
doi:10.12688/f1000research.2-102.v1 (2013).
- 544 Chiang, N. *et al.* Anesthetics impact the resolution of inflammation. *PLoS One* **3**, e1879,
doi:10.1371/journal.pone.0001879 (2008).
- 545 Demicheli, R. *et al.* Local recurrences following mastectomy: support for the concept of tumor
dormancy. *J Natl Cancer Inst* **86**, 45-48 (1994).
- 546 Dillekas, H. *et al.* The recurrence pattern following delayed breast reconstruction after
mastectomy for breast cancer suggests a systemic effect of surgery on occult dormant
micrometastases. *Breast Cancer Res Treat* **158**, 169-178, doi:10.1007/s10549-016-3857-1
(2016).
- 547 Karagiannis, G. S. *et al.* Neoadjuvant chemotherapy induces breast cancer metastasis through a
TMEM-mediated mechanism. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aan0026 (2017).
- 548 Volk-Draper, L. *et al.* Paclitaxel therapy promotes breast cancer metastasis in a TLR4-dependent
manner. *Cancer Res* **74**, 5421-5434, doi:10.1158/0008-5472.CAN-14-0067 (2014).
- 549 Chang, Y. S., Jalgaonkar, S. P., Middleton, J. D. & Hai, T. Stress-inducible gene Atf3 in the
noncancer host cells contributes to chemotherapy-exacerbated breast cancer metastasis. *Proc*
Natl Acad Sci U S A **114**, E7159-E7168, doi:10.1073/pnas.1700455114 (2017).
- 550 de Ruiter, J., Cramer, S. J., Smink, T. & van Putten, L. M. The facilitation of tumour growth in the
lung by cyclophosphamide in artificial and spontaneous metastases models. *Eur J Cancer* **15**,
1139-1149 (1979).
- 551 Orr, F. W., Adamson, I. Y. & Young, L. Promotion of pulmonary metastasis in mice by bleomycin-
induced endothelial injury. *Cancer Res* **46**, 891-897 (1986).
- 552 Ormerod, E. J., Everett, C. A. & Hart, I. R. Enhanced experimental metastatic capacity of a human
tumor line following treatment with 5-azacytidine. *Cancer Res* **46**, 884-890 (1986).
- 553 Abubaker, K. *et al.* Short-term single treatment of chemotherapy results in the enrichment of
ovarian cancer stem cell-like cells leading to an increased tumor burden. *Mol Cancer* **12**, 24,
doi:10.1186/1476-4598-12-24 (2013).
- 554 Gunjal, P. M. *et al.* Evidence for induction of a tumor metastasis-receptive microenvironment for
ovarian cancer cells in bone marrow and other organs as an unwanted and underestimated side
effect of chemotherapy/radiotherapy. *J Ovarian Res* **8**, 20, doi:10.1186/s13048-015-0141-7
(2015).
- 555 Karagiannis, G. S., Condeelis, J. S. & Oktay, M. H. Chemotherapy-induced metastasis in breast
cancer. *Oncotarget* **8**, 110733-110734, doi:10.18632/oncotarget.22717 (2017).
- 556 Sahu, R. P. *et al.* Chemotherapeutic agents subvert tumor immunity by generating agonists of
platelet-activating factor. *Cancer Res* **74**, 7069-7078, doi:10.1158/0008-5472.CAN-14-2043
(2014).
- 557 Leopold, W. R., Batzinger, R. P., Miller, E. C., Miller, J. A. & Earhart, R. H. Mutagenicity,
tumorigenicity, and electrophilic reactivity of the stereoisomeric platinum(II) complexes of 1,2-
diaminocyclohexane. *Cancer Res* **41**, 4368-4377 (1981).

- 558 Huang, Q. *et al.* Caspase 3-mediated stimulation of tumor cell repopulation during cancer
radiotherapy. *Nat Med* **17**, 860-866, doi:10.1038/nm.2385 (2011).
- 559 Chaurio, R. *et al.* UVB-irradiated apoptotic cells induce accelerated growth of co-implanted
viable tumor cells in immune competent mice. *Autoimmunity* **46**, 317-322,
doi:10.3109/08916934.2012.754433 (2013).
- 560 Ford, C. A. *et al.* Oncogenic properties of apoptotic tumor cells in aggressive B cell lymphoma.
Curr Biol **25**, 577-588, doi:10.1016/j.cub.2014.12.059 (2015).
- 561 da Silva, I. A., Jr., Chammas, R., Lepique, A. P. & Jancar, S. Platelet-activating factor (PAF)
receptor as a promising target for cancer cell repopulation after radiotherapy. *Oncogenesis* **6**,
e296, doi:10.1038/oncsis.2016.90 (2017).
- 562 Kornbluth, R. S. The immunological potential of apoptotic debris produced by tumor cells and
during HIV infection. *Immunol Lett* **43**, 125-132 (1994).
- 563 de Jong, J. S., van Diest, P. J. & Baak, J. P. Number of apoptotic cells as a prognostic marker in
invasive breast cancer. *Br J Cancer* **82**, 368-373, doi:10.1054/bjoc.1999.0928 (2000).
- 564 Naresh, K. N., Lakshminarayanan, K., Pai, S. A. & Borges, A. M. Apoptosis index is a predictor of
metastatic phenotype in patients with early stage squamous carcinoma of the tongue: a
hypothesis to support this paradoxical association. *Cancer* **91**, 578-584 (2001).
- 565 Jalalinadoushan, M., Peivareh, H. & Azizzadeh Delshad, A. Correlation between Apoptosis and
Histological Grade of Transitional Cell Carcinoma of Urinary Bladder. *Urol J* **1**, 177-179 (2004).
- 566 Sun, B. *et al.* Extent, relationship and prognostic significance of apoptosis and cell proliferation
in synovial sarcoma. *Eur J Cancer Prev* **15**, 258-265, doi:10.1097/01.cej.0000198896.02185.68
(2006).
- 567 Gregory, C. D. & Pound, J. D. Cell death in the neighbourhood: direct microenvironmental effects
of apoptosis in normal and neoplastic tissues. *J Pathol* **223**, 177-194, doi:10.1002/path.2792
(2011).
- 568 Gregory, C. D., Ford, C. A. & Voss, J. J. Microenvironmental Effects of Cell Death in Malignant
Disease. *Adv Exp Med Biol* **930**, 51-88, doi:10.1007/978-3-319-39406-0_3 (2016).
- 569 Alcaide, J. *et al.* The role and prognostic value of apoptosis in colorectal carcinoma. *BMC Clin
Pathol* **13**, 24, doi:10.1186/1472-6890-13-24 (2013).
- 570 Ichim, G. & Tait, S. W. A fate worse than death: apoptosis as an oncogenic process. *Nat Rev
Cancer* **16**, 539-548, doi:10.1038/nrc.2016.58 (2016).
- 571 Himuro, T. *et al.* Activated Caspase 3 Expression in Remnant Disease After Neoadjuvant
Chemotherapy May Predict Outcomes of Breast Cancer Patients. *Ann Surg Oncol* **23**, 2235-2241,
doi:10.1245/s10434-016-5164-9 (2016).
- 572 Flanagan, L. *et al.* Low levels of Caspase-3 predict favourable response to 5FU-based
chemotherapy in advanced colorectal cancer: Caspase-3 inhibition as a therapeutic approach.
Cell Death Dis **7**, e2087, doi:10.1038/cddis.2016.7 (2016).
- 573 Youlden, D. R. & Baade, P. D. The relative risk of second primary cancers in Queensland,
Australia: a retrospective cohort study. *BMC Cancer* **11**, 83, doi:10.1186/1471-2407-11-83
(2011).
- 574 Mariotto, A. B., Rowland, J. H., Ries, L. A., Scoppa, S. & Feuer, E. J. Multiple cancer prevalence: a
growing challenge in long-term survivorship. *Cancer Epidemiol Biomarkers Prev* **16**, 566-571,
doi:10.1158/1055-9965.EPI-06-0782 (2007).
- 575 Travis, L. B. The epidemiology of second primary cancers. *Cancer Epidemiol Biomarkers Prev* **15**,
2020-2026, doi:10.1158/1055-9965.EPI-06-0414 (2006).
- 576 Murphy, J. D. *et al.* Increased risk of additional cancers among patients with gastrointestinal
stromal tumors: A population-based study. *Cancer* **121**, 2960-2967, doi:10.1002/cnrc.29434
(2015).

- 577 Pandurengan, R. K. *et al.* Survival of patients with multiple primary malignancies: a study of 783 patients with gastrointestinal stromal tumor. *Ann Oncol* **21**, 2107-2111, doi:10.1093/annonc/mdq078 (2010).
- 578 Agaimy, A., Wunsch, P. H., Sobin, L. H., Lasota, J. & Miettinen, M. Occurrence of other malignancies in patients with gastrointestinal stromal tumors. *Semin Diagn Pathol* **23**, 120-129 (2006).
- 579 Miettinen, M., Kraszewska, E., Sobin, L. H. & Lasota, J. A nonrandom association between gastrointestinal stromal tumors and myeloid leukemia. *Cancer* **112**, 645-649, doi:10.1002/cncr.23216 (2008).
- 580 Davis, E. J., Beebe-Dimmer, J. L., Yee, C. L. & Cooney, K. A. Risk of second primary tumors in men diagnosed with prostate cancer: a population-based cohort study. *Cancer* **120**, 2735-2741, doi:10.1002/cncr.28769 (2014).
- 581 Tyzzer, E. E. Factors in the Production and Growth of tumor Metastases. *J Med Res* **28**, 309-332 301 (1913).
- 582 Fisher, E. R. & Fisher, B. Experimental Studies of Factors Influencing the Development of Hepatic Metastases. Xiii. Effect of Hepatic Trauma in Parabiotic Pairs. *Cancer Res* **23**, 896-900 (1963).
- 583 Ben-Eliyahu, S. The price of anticancer intervention. Does surgery promote metastasis? *Lancet Oncol* **3**, 578-579 (2002).
- 584 Coffey, J. C. *et al.* Excisional surgery for cancer cure: therapy at a cost. *Lancet Oncol* **4**, 760-768 (2003).
- 585 Goldstein, M. R. & Mascitelli, L. Surgery and cancer promotion: are we trading beauty for cancer? *QJM* **104**, 811-815, doi:10.1093/qjmed/hcr039 (2011).
- 586 O'Leary, D. P., Wang, J. H., Cotter, T. G. & Redmond, H. P. Less stress, more success? Oncological implications of surgery-induced oxidative stress. *Gut* **62**, 461-470, doi:10.1136/gutjnl-2011-300948 (2013).